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Optimisation of lipase production from olive pomace by solid-state fermentation
Felisbela Maria Araújo de Oliveira
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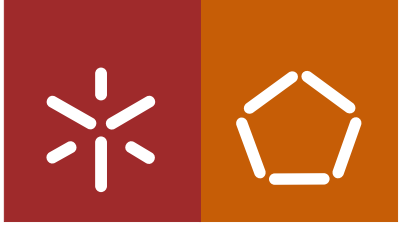

Universidade do Minho
Escola de Engenharia

Felisbela Maria Araújo de Oliveira

Optimisation of lipase production from olive pomace by solid-state fermentation

Doctoral Thesis in Chemical and Biological Engineering

Work developed under supervision of:
Doctor Isabel Maria Pires Belo
Doctor Armando Albino Dias Venâncio

March 2017

DECLARAÇÃO

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Optimisation of lipase production from olive pomace by solid-state fermentation

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STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, March 27th 2017

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“All of the efforts you make
will become a treasure in your life
– a treasure that will last a lifetime,
a treasure of happiness.”

DAISAKU IKEDA

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ABSTRACT

Agro-industrial residues are an excellent opportunity for biotechnological valorisation. Since they contain organic matter, carbohydrates, lipids, proteins, etc., they can be applied as substrates for solid-state fermentation (SSF) to produce added-value compounds. In particular, olive pomace (OP), a by-product from olive oil production, mainly obtained in Mediterranean countries, represents an environmental issue but also a renewable and low-cost resource to be explored. Its use for lipase production by fungi is an effective and eco-friendly solution as demonstrated by this work.

Initially, OP was applied on SSF mixed with wheat bran (WB) to produce lipase by *Aspergillus* species at Erlenmeyer´s flasks. Among three *aspergilli* used, *Aspergillus ibericus* MUM 03.49 presented the highest lipase production. Optimum SSF conditions of 60% moisture content (MC), 30 °C, 1.33% (w/w) $(NH_4)_2SO_4$ and ratio OP:WB of 1:1 (w/w, dry basis), giving C/N ratio of 25, were found. The mixture of OP with WB at 1:1 ratio is very convenient, since residues may be applied without pre-treatment and MC adjustment (the mixture origins around 60% MC), reducing the water consumption. A SSF was reproduced at lab-scale packed-bed bioreactor and an optimum aeration rate of 0.05 mL min^{-1} was found, using 25 g dry solid substrate.

Lipase production at optimum SSF conditions achieved was successfully scaled-up to tray-type and pilot-scale pressure bioreactors. Continuous pressurised aeration at 200 kPa was beneficial for lipase and for specific activity, in comparison to the traditional tray-type bioreactor. Lipase was characterised and maximum activity was found at 50 °C and pH 7.0. Enzymatic extracts were lyophilised as a way to preserve lipase, and an activity of 1000 U g^{-1} of lyophilised extract was obtained. The fermented substrate was further characterised and an increase of nutritional value was found, indicating a possible final application of solid as animal feed.

Lipase production was also performed using different oil cakes (OCs) from Brazilian agro-industries. Maximum lipase yields were found using a combination of 0.45 g palm kernel oil cake (PKOC) per g total substrate, mixed with sesame oil cake (SOC), 57% MC, and 1% NH_4Cl. At optimum conditions, a lipase production of 460 U g^{-1} was obtained after 6 days of SSF, and doubled in relation to SSF using OP+WB. Lyophilised fermented substrate containing lipase was further applied in esterification reactions, and the production of butyl decanoate ester was optimised. This ester has potential application in food industry.

RESUMO

Os resíduos agro-industriais são uma excelente oportunidade para valorização biotecnológica. Contêm matéria orgânica, carbohidratos, lípidos, proteínas, etc., e podem ser usados como substratos para fermentação em estado sólido (SSF) para produzir compostos de valor acrescentado. Em particular, o bagaço de azeitona, um subproduto da produção de azeite, maioritariamente obtido nos países Mediterrâneos, representa um problema ambiental mas também uma fonte renovável e de baixo custo a ser explorada. O seu uso para a produção de lipase por fungos é uma solução efetiva e amiga do ambiente, como demonstrado neste trabalho.

Inicialmente, o bagaço de azeitona foi usado para SSF misturado com farelo de trigo (WB) para produzir lipase por espécies de *Aspergillus* em frascos de Erlenmeyer. Das três espécies *aspergilli* estudadas, *Aspergillus ibericus* MUM 03.49 apresentou a maior produção. As condições ótimas para produção de lipase por SSF, foi de 60% de humidade (MC), 30 °C, 1.33% (m/m) $(NH_4)_2SO_4$ e razão de 1:1 de OP:WB (m/m, base seca), originando uma razão C/N de 25. A mistura de OP:WB na razão 1:1 origina cerca de 60% de humidade, o que permite que os resíduos possam ser usados sem pré-ajuste de MC. Os ensaios de SSF foram reproduzidos num biorreator de leito empacotado à escala laboratorial, tendo-se obtido como condição ótima de arejamento, um caudal de ar de 0.05 mL min^{-1}, usando 25 g de substrato seco.

A produção de lipase nas condições otimizadas de SSF foi reproduzida em maior escala em biorreatores tipo bandeja e de pressão. O arejamento contínuo sob pressão a 200 kPa foi benéfico na atividade de lipase e específica, tendo-se obtido melhores resultados que no biorreator tipo bandeja. A lipase foi caracterizada e apresentou atividade máxima a 50 °C e a pH 7.0. Os extratos enzimáticos foram liofilizados, como forma de preservar a lipase, e foi obtida uma atividade de 1000 U g^{-1} no extrato liofilizado. O substrato fermentado foi caracterizado, e verificou-se um aumento do valor nutricional, possibilitando a sua aplicação final em alimentação animal.

A produção de lipase foi também realizada em tortas de óleo, de agro-indústrias do Brasil. A produção máxima de lipase foi obtida usando a combinação de 0.45 g de torta de óleo de dendê (PKOC) por g de substrato total, misturado com torta de sésamo (SOC), 57% MC, e 1% (m/m) de NH_4Cl. Nas condições ótimas, obteve-se uma produção de 460 U g^{-1} de lipase, após 6 dias de SSF, e foi o dobro em relação à SSF usando OP+WB. O substrato fermentado liofilizado contendo lipase foi aplicado em reações de esterificação, e a produção do éster decanoato de butilo foi otimizada. Este éster tem potencial aplicação na indústria alimentar.

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LIST OF ABBREVIATIONS

a	Coefficient, intercept
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADF	Acid detergent fibre
AOC	Andiroba oil cake
CaOC	Canola oil cake
CNR	C/N ratio
CrOC	Crambe oil cake
CuOC	Cupuassu oil cake
C4:0	Glyceryl tributyrate
C8:0	Glyceryl trioctanoate
C10:0	Glyceryl tridecanoate
C16:0	Glyceryl tripalmitate
C18:0	Glyceryl triesterate
df	Degrees of freedom
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FAAS	Flame atomic absorption spectrometry
FAES	Flame atomic emission spectrometry
FDA	Food and Drug Administration
FFA	Free fatty acid
GCOC	Green coffee oil cake
GRAS	Generally Recognised as Safe
LA	Lipase activity (U g^{-1} or U mL^{-1})
Log *P*	Logarithm of the partition coefficient
LP	Lipids concentration (%, w/w)

LR	Lipase recovery (%)
MOC	Macauba oil cake
MS	Mean squares
NDF	Neutral detergent fibre
OC	Oil cake
OP	Olive pomace
OTA	Ochratoxin A
PA	Protease activity (U g^{-1})
PC	Principal component
PCA	Principal components analyses
pNP	*p*-nitrophenol
pNPB	*p*-nitrophenyl butyrate
pNPG	4-nitrophenyl β-D-glucopyranoside
PKOC	Palm kernel oil cake
QAH	Quantitative acid hydrolysis
R^2	Coefficient of determination
RPKOC	Ratio of palm kernel oil cake (g g^{-1})
RS	Reducing sugars concentration (%, w/w)
SA	Specific activity (U mg^{-1})
SBM	Soybean meal
SD	Standard deviation
SI	International System of Units
SmF	Submerged fermentation
SOC	Sesame oil cake
SS	Sum of squares

SSF Solid-state fermentation

T Temperature (°C)

TAG Triacylglycerol

WB Wheat bran

X/R Characteristic increase (or reduction)

X Characteristic

x Independent variable

y Predicted response

Subscripts

i Initial

f Final

1 Independent variable

2 Independent variable

Greek letters

ε Molar extinction coefficient (mM^{-1})

Remarks

In general, the International System of Units (SI) was used in this work. Sometimes multiples and sub-multiples of the SI units were also used, as well as other no-SI units but allowed by SI, as the use of litre to express volume.

Some units not recognised by the SI were also used to express some variables, such as the ratio or volume percent (%, v/v), mass percent (%, w/w) and mass per volume percent (%, w/v), to denote the composition of some solutions, and the revolutions per minute (rpm) to indicate the agitation rate.

1 Motivation and outline

This chapter introduces the background information about the theme of the work, as well as its objectives.

The outline of the thesis and its outputs are also presented.

1.1 Context and motivation

Mediterranean countries, led by Spain, produce 93% of the world olive oil. Portugal, contributing with 2.3% of the Mediterranean countries olive oil production, produced around 66.5 thousand tonnes of olive oil in 2014 (FAO, 2016). Therefore, environmentally sound disposal of olive mill wastes during a short harvesting period is a priority in the agenda of these countries. Olive oil is extracted from olives by physical methods. Olive crushing/malaxing and oil phase separation are the most important stages of the extraction process (Azbar et al., 2004). Currently, these operating steps are mainly carried out by two types of continuous processes centrifugation: three-phase system, which separates olive pulp into oil, liquid wastes (olive mill wastewaters (OMWW)) and solid residue; and two-phase system, which separates olive pulp into oil and wet solid residue (Aliakbarian et al., 2011). The new two-phase system replaced recently the three-phase system process, since it reduces water needs and wastewaters (Azbar et al., 2004). The two-phase process generates olive pomace (OP), representing 80% (w/w) of the olive weight processed (Azbar et al., 2004). OP contains 4 - 18% (w/w) fats and 50 - 71% moisture content (MC) (Roig et al., 2006). Currently in Portugal, 84% of the olive oil is produced by the two-phase process (INE, 2016). However, a lack of OP valorisation solutions still exists. Currently, a second extraction of the remaining pomace oil prior to combustion is the main disposal option (Roig et al., 2006).

As many other agro-industrial residues, OP offers excellent possibilities to be used for enzyme production by solid-state fermentation (SSF) by filamentous fungi. Particularly for lipase production, OP is promising due to its residual content in olive oil. Recently, a growing interest on lipase production in low-cost agro-industrial residues has emerged, and the use of substrates such as wheat bran (WB) has been proposed (Damaso et al., 2008), where the addition of the inducer olive oil has proven to improve lipase productivity. This step will not be needed using OP available in Portugal. On the other hand, the overall market for industrial enzymes is still increasing. Lipases are considered the third largest group, followed by proteases and carbohydrases, based on total sales volume (Hasan et al., 2006). Despite the many applications of lipases, such as in detergents industry, food industry, biodiesel production, among others, the challenge is to reduce the production costs to make the lipase use profitable compared to chemical processes. Accordingly, SSF can be a potential process to reduce the costs, unlike submerged fermentation (SmF), since it can be used low-cost substrates such as OP and energy costs are minor. It has been shown that

capital investment for SmF in large-scale is 78% higher than for SSF (Castilho et al., 2000) and higher concentration and stability of the enzyme is usually obtained in SSF (Longo et al., 2008).

The main goal of this thesis consisted in the development of strategies to improve lipase production by *Aspergillus ibericus* using OP as substrate. Various parameters were considered, as MC, temperature, substrates ratio, additional nitrogen source, to optimise lipase production. Also different bioreactors were tested in this bioprocess: lab-scale packed-bed, tray-type and pilot-scale pressure bioreactors. Lipase was further characterised and the fermented substrate was evaluated for animal feed. Additionally, the use of oil cakes (OCs) as substrates for SSF to produce lipase and optimisation of process was also achieved. Lipase produced was applied in esterification reactions to produce an aroma ester.

1.2 Outline of the thesis

The thesis was structured in eight chapters.

Chapter 1 (current chapter) presents the context, motivation and the research goals of this Ph.D. thesis. The structure of the thesis, the stays and the scientific outputs of the thesis are also outlined.

In chapter 2 is presented the literature review of the state of art of the biotechnological production of metabolites, as lipase, through SSF of agro-industrial residues, the optimisation of the bioprocess and scale-up issues.

Experimental results are presented from chapter 3 to chapter 6. In these chapters, an abstract, introduction, material and methods, results and discussion, and conclusions are provided.

Chapter 3 presents lipase production by SSF of OP using three *Aspergillus* species. Parameters as MC and temperature were studied in order to optimise lipase production.

In chapter 4 the optimisation of *A. ibericus* lipase production by SSF of OP in flasks and in a lab-scale packed-bed bioreactor is described. Variables such as source of nitrogen and composition of mixtures of OP and WB were studied. Also, the effect of aeration rate on lipase in a packed-bed bioreactor was evaluated. Additionally, lipase extraction conditions were optimised.

Chapter 5 describes the scale-up process of SSF of OP for lipase production at tray-type and pilot-scale pressure bioreactors. *A. ibericus* lipase was further characterised in terms of the relation of activity with temperature and pH, and also the impact of lyophilisation as a way to preserve lipase on enzyme activity. In addition, the fermented substrate was chemically characterised to determine its applicability as animal feed.

In chapter 6 is presented the optimisation of *A. ibericus* lipase production by SSF using OCs from Brazil, and the application of lipase produced in esterification reactions.

Chapter 7 presents the overall conclusions of the thesis and suggestions for the future work.

Chapter 8 gathers all the references used in the elaboration of this Ph.D. thesis.

1.3 Stays in international universities

The work performed in a packed-bed and pressurised bioreactors, presented in chapter 4 and chapter 5, was performed at CITI, University of Vigo, Ourense, Spain, under the supervision of Doctor José Manuel Domínguez, during 6 months.

The work performed using OCs, presented in chapter 6, was performed at Centre of Technology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, under the supervision of Doctor Maria Alice Zarur Coelho, during 6 months.

1.4 Outputs of the thesis

According to the 2nd paragraph of the article 8 of the Portuguese Decree-Law no. 388/70, the scientific outputs of this thesis are listed below.

1.4.1 Articles published in scientific journals

Oliveira, F., Pérez-Rodríguez, N., Domínguez, J. M., Venâncio, A., & Belo, I. (2017). Scale-up of lipase production by solid-state fermentation of olive pomace in tray-type and pilot-scale pressure bioreactors. *Bioresource Technology* (Submitted).

Oliveira, F., Salgado, J. M., Abrunhosa, L., Pérez-Rodríguez, N., Domínguez, J. M., Venâncio, A., & Belo, I. (2017). Optimization of lipase production by solid-state fermentation of olive pomace: from flask to lab-scale packed-bed bioreactor. *Bioprocess and Biosystems Engineering* (Accepted).

Oliveira, F., Souza, C. E., Peclat, V. R. O. L., Salgado, J. M., Ribeiro, B. D., Coelho, M. A. Z., Venâncio, A., & Belo, I. (2017). Optimization of lipase production by *Aspergillus ibericus* from oil cakes and its application in esterification reactions. *Food and Bioproducts Processing,* 102, 268–277.

Oliveira, F., Moreira, C., Salgado, J. M., Abrunhosa, L., Venâncio, A., & Belo, I. (2016). Olive pomace valorization by *Aspergillus* species: lipase production using solid-state fermentation. *Journal of the Science of Food and Agriculture*, 96, 3583–3589.

The work of this thesis was presented in national and international conferences, as poster, oral presentations and publications in Conference´s Proceedings. Works presented at these events are listed below.

1.4.2 Oral presentations and publications in Conference´s Proceedings

Oliveira, F., Souza, C. E., Ribeiro, B. D., Peclat, V. R. O. L., Coelho, M. A., Venâncio, A., & Belo, I. (2016). Lipase production by *Aspergillus ibericus* using oil cakes and its application in esterification reactions. In *Book of Proceedings of WasteEng2016 - 6th International Conference on Engineering for Waste and Biomass Valorisation,* pp. 964-971. ISBN: 979-10-91526-05-0. Albi, France, 23-26 May.

Oliveira, F., Venâncio, A., Belo, I., Pérez-Rodríguez, N., & Domínguez, J. M. (2015). Scale-up of *Aspergillus ibericus* lipase production by solid-state fermentation. In C. Vilarinho, F. Castro, & M. Russo (Eds.), *Wastes: Solutions, Treatments and Opportunities* (Taylor & Francis Group), pp. 203–208. London. ISBN: 978-1-138-02882-1. (Selected papers from the 3rd edition of the International Conferences on Wastes: Solutions, Treatments and Opportunities, Viana do Castelo, Portugal, 14-16 September).

Oliveira, F., Abrunhosa, L., Venâncio, A., Belo, I., Pérez-Rodríguez, N., & Domínguez, J. M. (2015). *Aspergillus ibericus* lipase production by solid-state fermentation of olive pomace. In C. Vilarinho, F. Castro, & M. Russo (Eds.), *Wastes: Solutions, Treatments and Opportunities* (Taylor & Francis Group), pp. 195–201. London. ISBN: 978-1-138-02882-1. (Selected papers from the 3rd edition of the International Conferences on Wastes: Solutions, Treatments and Opportunities, Viana do Castelo, Portugal, 14-16 September).

1.4.3 Flash presentation plus poster presentation

Oliveira, F., Salgado, J. M., Abrunhosa, L., Venâncio, A., & Belo, I. (2013). Influence of moisture content, temperature and inoculum size on lipase production by filamentous fungi under solid-state fermentation of olive pomace. In *Book of Abstracts of MicroBiotec´13 – Portuguese Congress of Microbiology and Biotechnology*, pp. 124. Aveiro, Portugal. 6-8 December.

2 Literature review

In the last decades it has increased the interest in solid-state fermentation (SSF) for the development of biotechnological processes, such as simultaneous biological detoxification of agro-industrial residues and added-value compounds production. Bioconversion of agro-industrial residues for lipase production as well as other added-value compounds holds a prominent position as an emergent biotechnology application.

This chapter discusses the main developments on the valorisation of agro-industrial residues by SSF and presents the main advantages of SSF in relation to submerged fermentation (SmF) technology. In SSF process, parameters such as substrate type and composition, moisture content (MC) and temperature must be optimised to maximise metabolite production. In addition, these parameters are important on SSF scale-up combined to bioreactor type and others operation conditions.

This chapter also addresses the availability and suitability of different agro-industrial residues obtained from processed crops, such as oil cakes (OCs) from oils production, to be applied in SSF for lipase production. Particularly, olive pomace (OP) obtained from olive mills, which presents an environmental problem in the Mediterranean region, due to its characteristics, constitutes a promising substrate to be valorised by SSF for enzymes production.

2.1 Solid-state fermentation

Solid-state fermentation (SSF) is defined as a fermentation process which occurs in absence of free water. However, solid substrate must possess moisture to support microorganisms growth (Pandey, 2003). SSF simulates the natural habitat of filamentous fungi and is the preferred choice for filamentous fungi's growth and for the production of added-value compounds. Submerged fermentation (SmF) can be considered as a violation to the natural habitat, especially for filamentous fungi (Singhania et al., 2009). The moisture content (MC) needed in SSF exists in absorbed or complex form within the solid matrix, which is likely more advantageous for growth, since oxygen transfer process may be more efficient. In SSF, the water content is quite low and the microorganism is almost in contact with gaseous oxygen in the air, unlike in the case of SmF (Raghavarao et al., 2003). Many microorganisms are capable of growing on solid substrate, but only filamentous fungi can grow to a significant extent in the absence of free water. Bacteria and yeasts grow on solid substrates at 40 - 70% MC level, such as composting, anaerobic and aerobic ensiling, but the growth and propagation of single cell organisms always require free water (Raghavarao et al., 2003).

SSF has found increased application in the production of antibiotics, surfactants, biocides and enzymes (Couto and Sanromán, 2005). SSF processes are known from ancient times in Asia. Typical examples of SSF are traditional food elaborations such as Japanese "koji", Indonesian "temph" and French "blue cheese" (Couto and Sanromán, 2005). SSF processes were completely ignored in western countries after 1940 due to the adaptation of SmF technology. The SmF technology has become the model fermentation technology for the production of any compound, as a result of advances introduced for the production of penicillin by SmF (Couto and Sanromán, 2005; Pandey, 2003). During 1960 - 1970, SSF slowly extends when reports appeared on mycotoxins production by SSF. Also, the production of proteins, involving the utilisation of agro-industrial residues for animal feed, was a major activity reported, offering the valorisation of low cost residues (Pandey, 2003).

2.1.1 SSF versus SmF

Most enzyme manufacturers produce enzymes by SmF. However, in the last decades there has been an increasing interest on SSF technique for enzymes production (Couto and Sanromán, 2005). In addition, SSF presents several advantages over SmF, as described on Table 2.1.

SSF appears to possess several biotechnological advantages, such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of microorganisms specialised for the water-insoluble substrates or mixed cultivation of various fungi, and lower demand on sterility due to the low water activity in SSF (Singhania et al., 2009). Nutrient availability is more restricted in natural solid substrates than in liquid cultures, therefore, it is likely that fungi have to develop more efficient enzyme systems for host cell degradation than in liquid cultures (Tengerdy and Szakacs, 2003).

Table 2.1 – Advantages and disadvantages of SSF over SmF (Couto and Sanromán, 2005, 2006).

Advantages	Disadvantages
Higher yields in a shorter period of time	Problems in scale-up
Better oxygen circulation	Low mix effectively
Low-cost media	Less knowledge of the SSF process
It resembles the natural habitat for filamentous fungi	Difficult control of process parameters (pH, heat, nutrient conditions, etc.)
Reduced energy and cost requirements	
Less effort in downstream processing	
Scarce operational problems	
Wild-type strains of microorganisms perform better in SSF than do genetically modified ones	

In SSF, the water content is quite low and microorganisms are almost in contact with gaseous oxygen in the air, unlike in the case of SmF (Raghavarao et al., 2003). SSF is often simpler and requires less processing energy than SmF. The low volume of water present in the media per unit of mass substrate can substantially reduce the space occupied by the fermenter, without sacrificing the product yield (Raghavarao et al., 2003). On the negative side, SSF processes

are slower than the liquid fermentations, due to the additional barrier from the bulk solid. They also present heat dissipation problems, which can be limited by inter and intraparticle resistances and are more difficult to control, due to the lack of adequate sensors and efficient solid handling techniques, especially for continuous operations (Raghavarao et al., 2003).

Scale-up of SSF processes has been a limiting factor. A number of bioreactors have been designed to overcome the associated problems of scale-up (Singhania et al., 2009). Several problems encounters in the control of different parameters such as pH, temperature, aeration, oxygen transfer and MC. SSF lacks the sophisticated control mechanisms that are usually associated with SmF. Control of the environment SSF within the bioreactors is also difficult to achieve, particularly temperature and MC (Couto and Sanromán, 2006).

2.2 Valorisation of agro-industrial residues

Food processing residues have long been considered as a matter of treatment, minimisation and prevention due to the environmental effects induced by their disposal (Galanakis, 2012). Food residues today are considered as a cheap source of valuable components, since the existent technologies allow the recovery of target compounds and their recycling inside food chain as functional additives in different products (Galanakis, 2012). SSF offers numerous opportunities in processing of agro-industrial residues. This is partly because solid-state processes have lower energy requirements, produce less wastewater and are environmental-friendly, as they resolve the problem of solids disposal (Pandey, 2003).

On the other hand, applications of agro-industrial residues in bioprocesses helps in solving pollution problems, which otherwise may cause their disposal (Pandey et al., 2000). Research on the selection of a suitable substrate has mainly centered on tropical agro-industrial crops and residues. These include crops (cassava, soybean, sugar beet, sweet potato, potato, and sweet sorghum); crop residues (bran and straw of wheat and rice, hull of soy, corn and rice); bagasse (sugarcane and cassava); coffee residues from processing industry (coffee pulp, coffee husk, coffee spent-ground); residues of fruit-processing industries (apple and grape pomace, pineapple and banana residues); wastes of oil-processing mill (coconut cake, soybean cake, peanut cake, canola meal and palm cake); and others such as corn cobs, carob pods, tea waste, chicory roots, etc. (Pandey et al., 2000).

Many processes have been developed for the utilisation of these residues for the production of added-value products such as ethanol, single-cell protein, mushrooms, enzymes, organic acids (Pandey et al., 2000; Raghavarao et al., 2003), biogas, antibiotics, surfactants, microbial polysaccharides, biocides, mycotoxins, etc. (Raghavarao et al., 2003). SSF can be used in a controlled way to produce the desired product (Singhania et al., 2009).

Enzymes such as phytase, amylase, inulinase, cellulase, protease, alpha-galactosidase, lipase, tannase, laccase, chitinase, L-glutaminase; organic acids such as lactic acid and citric; antibiotics such as cephamycin C; and other metabolites as bioethanol, polyunsatured acid, iturin A, pigment, hexyl laureate and palatinose, have been successfully produced employing SSF (Singhania et al., 2009). Couto and Sanromán (2005) compiled the different agro-industrial residues used to produce lignocellulolytic enzymes, and it is presented in Table 2.2.

Table 2.2 – Agro-industrial residues used to produce lignocellulolytic enzymes by SSF (Couto and Sanromán, 2005).

Substrate	Lignocellulolytic enzymes
Ballico seed	Laccase
Banana waste	Laccase, lignin peroxidase
Canola roots	Laccase, manganese peroxidase
Corn	Laccase
Cotton	Cellulase, laccase, manganese peroxidase
Grape	Laccase, lignin peroxidase, manganese peroxidase, glycosidase
Sugarcane bagasse	Laccase, lignin peroxidase, manganese peroxidase
Wheat bean	Manganese peroxidase
Wheat bran	Laccase, lignin peroxidase, manganese peroxidase
Wheat straw	Laccase, lignin peroxidase, manganese peroxidase

The substrate selection for SSF processes depends upon several factors mainly related with cost and availability; and thus may involve screening of several agro-industrial residues (Pandey et al., 2000). Table 2.3 presents some examples of applications of substrates for the production of added-value compounds. Moreover, the support should be selected attending to the enzyme which is going to be produced, since it may contain compounds acting as inducers for such enzyme (Couto and Sanromán, 2005). As examples, the production of lignin peroxidase was favoured by the utilisation of organic residues rich in lignin, and the use of rice led to the production of

amylase, since contain high amount of starch (Couto and Sanromán, 2005). Lipidic carbon sources generally seem to be essential to obtain a high lipase yield; whereas, a few authors observed that the presence of fats and oils was not statistically significant for enzyme production (Contesini et al., 2010).

Table 2.3 – Examples of substrates and application of SSF for the production of added-value compounds (Bellon-Maurel et al., 2003).

Substrate	Application
Lignocellulosic	
Wheat straw	Protein enrichment
	Mushroom cultivation
Wheat bran	Enzyme production
	Protease production
	Flavour production
Sugarcane bagasse	Protein enrichment
	Penicillin production
	L-lactic acid production
Sugar beet pulp	Protein enrichment for feed
Coffee pulp	Pectinase production
Coffee husk	Citric acid production
	Flavour production
Starchy	
Rice bran	Aroma production
	Protease production
	Pigment production
Cassava bagasse	Flavour production
Buckwheat seeds	Fungal spore production
Soybean	Fermented food production
Banana wastes	Protein enrichment for feed
Others	
Apple pomace	Ethanol production
Kiwifruit peel	Citric acid production
Amaranth grain	Volatile compound production

2.2.1 Oil industry residues

According to the Food and Agriculture Organization (FAO), in 2014, 173 billons tonnes of oils and 14 billons tonnes of short margarine were produced, where oil palm had the highest production in the world (57 billons tonnes), but it was not produced in Mediterranean countries. Contrariwise, olive is produced mainly in Mediterranean countries, representing 93% of the total production in the world, with a production of around 3 billons tonnes of olive oil in 2014 (FAO, 2016). Figure 2.1 presents the processed crops (oils and margarine) in the world and Mediterranean during the year 2014. In Portugal, olive oil is the third most produced oil, after soybean and sunflower oils (Figure 2.2), where, in 2014, 66.5 thousand tonnes were produced (FAO, 2016).

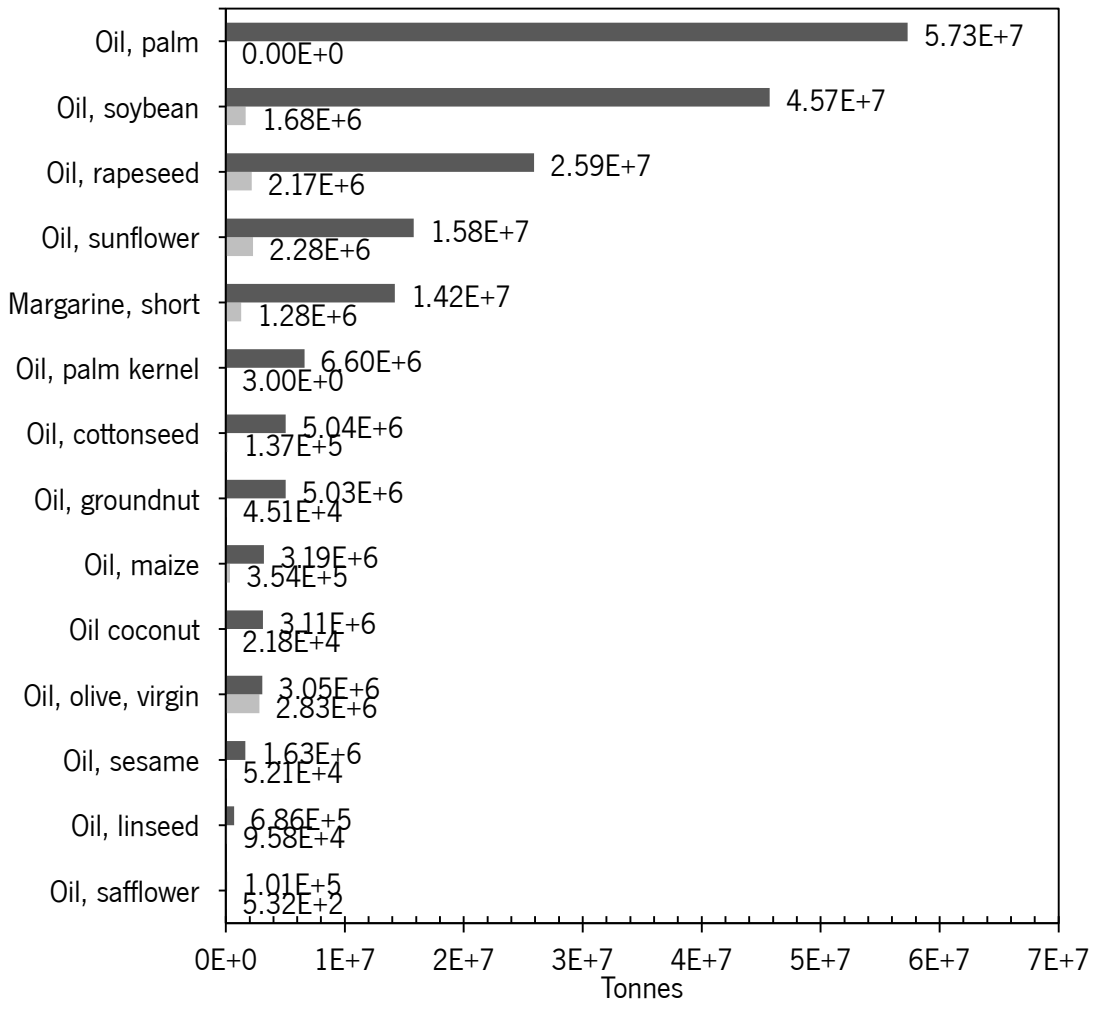


Figure 2.1 – Production, in tonnes, of processed crops (oils and margarine) in (■) the world and (■) Mediterranean, in 2014 (FAO, 2016).

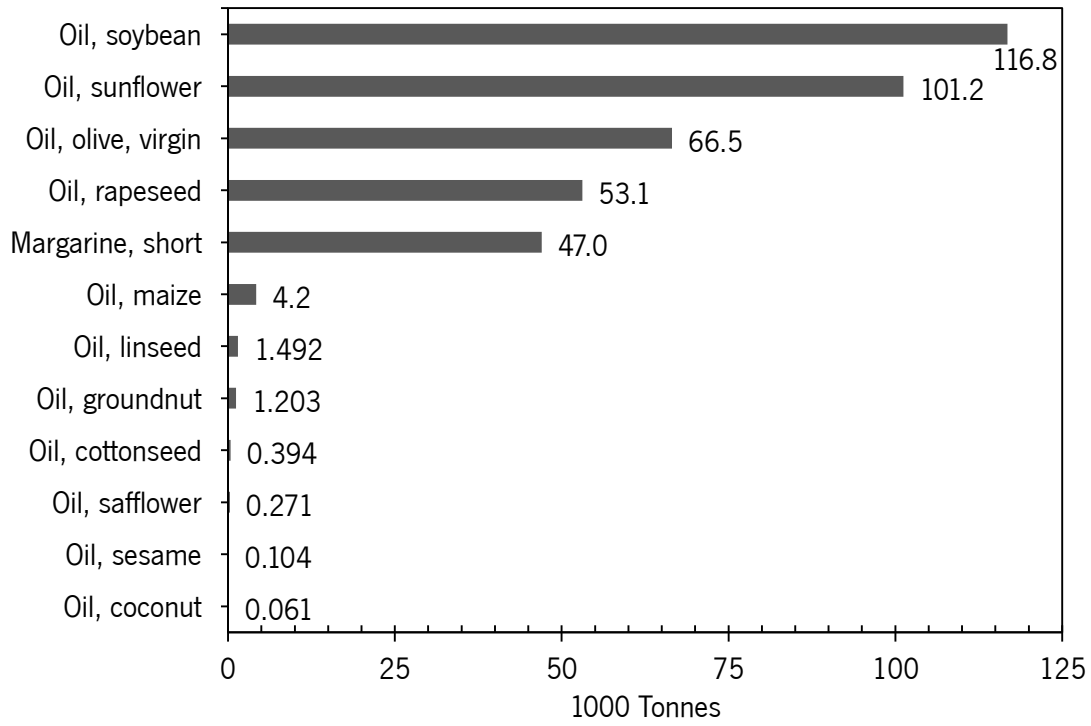


Figure 2.2 – Production, in tonnes, of processed crops (oils and margarine) in Portugal in 2014 (FAO, 2016).

The extraction of the oil from crops or seeds cultures originates agro-industrial by-products, called oil cakes (OCs) (Ramachandran et al., 2007). Their composition vary depending in their variety, growing conditions and extraction methods, and can be classified as edible or non-edible OCs (Singhania et al., 2008). The main application of OCs is as animal feed, due to their high protein content. They have been used as feed in poultry, fish and swine industry (Singhania et al., 2008). However, several OCs have low protein content and are considered non-edible oil cakes. Thus, OCs can be used in other applications, such as the production of antibiotics and biopesticides (Kota and Sridhar, 1999; Sircar et al., 1998) and other biochemicals (Ohtsuki et al., 2003), in the production of mushrooms (Bano et al., 1993), as a bio-control agent (Khan and Saxena, 1997), and can also be used for energy production (Atimtay and Topal, 2004). OCs are a good source of carbon, nitrogen and other compounds (Ramachandran et al., 2007). Thus, they are considered good substrates for fermentation processes as SSF. Since OCs may contain residual oil, they can be used as substrate for lipase production. Table 2.4 shows some important applications of OCs in bioprocesses.

Table 2.4 – Application of oil cakes in bioprocesses for metabolites production (Ramachandran et al., 2007).

Oil cake	Product production
Canola oil cake (CaOC)	Phytase
Cottonseed oil cake (CSOC)	Cephamycin C, glucoamylase, mushroom cultivation
Coconut oil cake (COC)	α-Amylase, endotoxin, glucoamylase, inulinase, lipase, phytase, protease
Groundnut oil cake (GOC)	α-Amylase
Mustard oil cake (MuOC)	Lactic acid, mushroom cultivation
Palm kernel oil cake (PKOC)	α-Amylase, mannanse tannase
Sesame oil cake (SOC)	L-Glutaminase, lipase, phytase
Sunflower oil cake (SuOC)	α-Amylase, cephamycin C, clavulanic acid, mushroom cultivation

2.2.2 Olive oil industry

Olive is a very popular oil crop in the Mediterranean region (Galanakis, 2012). Spain is the major virgin olive oil producer, where, in 2014, 1.7 billons tonnes were produced, followed by Italy (around 300 000 tonnes), Greece (210 000 tonnes), Tunisia (180 000 tonnes), Morocco (140 000 tonnes) and Portugal (66 500 tonnes) (FAO, 2016). Olive mill wastes are agro-industrial residues which are obtained from olive oil extraction and represent an environmental problem in Mediterranean countries. They are produced in large quantities in short periods of time (3 - 4 months) and must be properly disposed, in order to avoid environmental risks (Arvanitoyannis and Kassaveti, 2007). Olive oil production is obtained by extraction process, which involves various steps as olive washing, crushing/malaxing and extraction of the oil (pressing or centrifugation) (Azbar et al., 2004). Extra virgin olive oil can be obtained by traditional press (discontinuous process) or centrifugation systems (continuous process), which consists in the three-phase and the two-phase continuous systems (Dermeche et al., 2013). In the three-phase system, olive oil is separated from two other by-products, olive mill wastewater and a solid residue; whereas, in the two-phase system, only a semi-solid waste is obtained, which contains both water and solid residue (Aliakbarian et al., 2011).

Table 2.5 – Outputs from olive oil production systems using 1000 kg olives (Azbar et al., 2004).

Outputs	Traditional	Three-phase system	Two-phase system
Olive oil (kg)	200	200	200
Olive cake (kg)	400	500 - 600	800 - 950
Wastewater (kg)	600	1000 - 1200	-

In the three-phase system, large amounts of liquid waste known as olive mill wastewater are produced (Table 2.5), which is a significant source of agro-industrial pollutants (Abrunhosa et al., 2013). The three-phase system is widely used in Italy, Greece and other Mediterranean countries (Aliakbarian et al., 2011). However, in Portugal and Spain the two-phase extraction system was largely implemented in the olive mills, turning olive pomace (OP) (solid fraction, also called olive cake) the most important by-product produced. Figure 2.3 presents the evolution of extraction systems in olive mills in the last years, in Portugal. Nowadays, the traditional pressing system is almost extinguished (Alburquerque et al., 2004). In Portugal, the two-phase system technology processes 84% of the olives for oil extraction, originating more than 350 thousand tonnes of OP, during the year 2015 (INE, 2016).

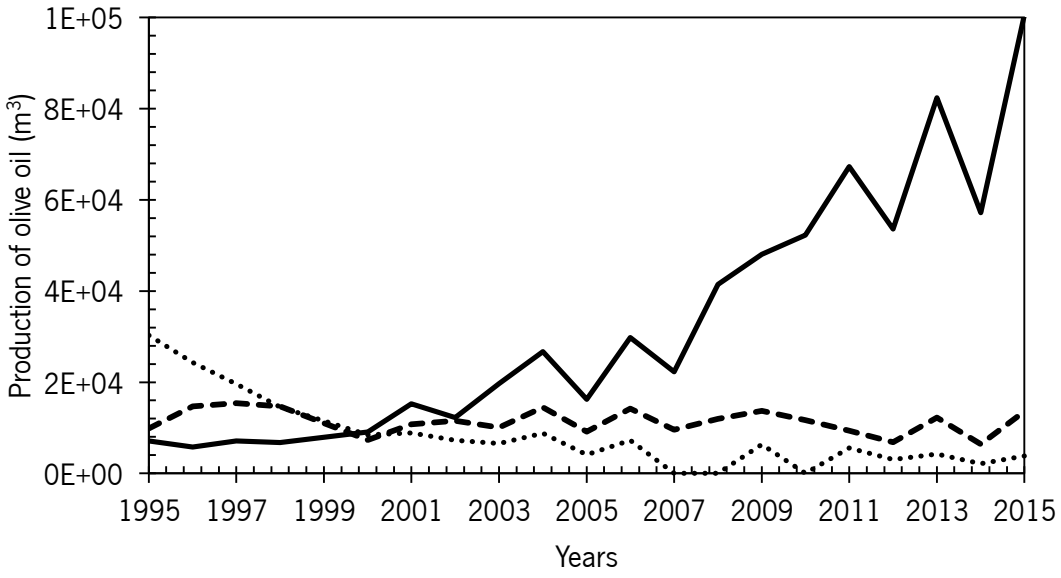


Figure 2.3 – Evolution of extraction systems in Portuguese olive mills industry over the years. Representation of (·····) traditional system, (– – – –) three-phase system and (——) two-phase system (INE, 2016).

Table 2.6 presents the olive oil produced in the different regions from Portugal and the respective system used, in 2015. OP is the main waste generated in the extraction of olive oil by two-phase extraction system. It causes more disposal problems than liquid wastes generated from the previous three-phase system. Currently, a second extraction of the remaining pomace oil prior to combustion is the main disposal option (Roig et al., 2006).

Table 2.6 – Production of olive oil (m^3) in agricultural regions of Portugal in 2015 (INE, 2016).

Region	Total	Traditional	Two-phase system	Three-phase system	Others
Alentejo	86 620	190	79 958	6 473	0
Trás-os-Montes	14 293	891	12 216	1 186	0
Ribatejo e Oeste	6 427	1 036	2 875	1 664	851
Beira Litoral	5 460	428	2 490	2 204	337
Beira Interior	5 158	1 150	2 350	1 653	5
Algarve	689	4	311	374	0
Entre Douro e Minho	406	89	297	20	0
Portugal	119 052	3 788	100 498	13 574	1 192

OP or also called olive wet cake, olive wet husk, alperujo or two-phase olive mill waste (TPOMW), is a solid waste with a strong odour and a doughy texture. OP is very humid material, it has a slightly acidic pH, a high organic matter concentration (mainly fibres). It presents high lipids concentration, depending on the extraction yield, and also contains carbohydrates and polyphenols (Roig et al., 2006). Table 2.7 shows range values of some chemical characteristics given by several authors. OP is a widely available and valueless residue which requires a proper treatment and valorisation. OP contains valuable compounds and it has been suggested the extraction of pectins and phenols. Pectins are natural hydrocolloids widely used as gelling agents, stabilizers and emulsifiers in the food industry. Phenols as hydroxytyrosol, tyrosol, oleuropein and caffeic acid are the major components in OP, which can be used in pharmaceutical, cosmetic and food industries (Roig et al., 2006). Since OP contains high organic matter, it is reported in the literature that OP may be applied in composting (Alburquerque et al., 2006a); production of hydrogen, methane and ethanol as biofuel (Ballesteros et al., 2001; Gavala et al., 2005); polymers (Ramos-Cormenzana et al., 1995) and enzymes, such as lignocellulolytic ones (Salgado et al., 2014a). Also, OP can be

used for lipase production due to the residual oil content (Oliveira et al., 2016; Salgado et al., 2014b). However, OP is characterised by a low N content (Alburquerque et al., 2004; Leite et al., 2016), thus their C/N ratio is not suitable for SSF and it should be corrected by the addition of a N source and/or a mixture of different solid substrates (Salihu et al., 2012).

Table 2.7 – Range values of main chemical characteristics of OP (Roig et al., 2006).

Characteristics	Range value
MC (%, w/w)	50 - 71
pH	4.9 - 6.8
N (g kg^{-1})	10 - 19
C/N	29 - 60
Lignin (%, w/w)	20 - 48
Hemicellulose (%, w/w)	15 - 39
Cellulose (%, w/w)	17 - 34
Lipids (%, w/w)	4 - 18
Proteins (%, w/w)	6.7 - 7.2
Carbohydrates (%, w/w)	10 - 19
Phenols (%, w/w)	0.5 - 2.4

2.3 Lipases

Enzymes are considered as nature`s catalysts. Most enzymes today (and probably nearly all in the future) are produced by the fermentation of biological materials. Lipids constitute a large part of the earth`s biomass, and lipolytic enzymes play an important role in the turnover of water-insoluble compounds (Hasan et al., 2006).

Lipases belong to the enzyme class of hydrolases (E.C.3). They act on ester bonds (E.C.3.1) of carboxylic esters (E.C.3.1.1). They hydrolyse triacylglycelors to fatty acids, diaglycerol, monoglycerol, and glycerol, and known as triacylglycerol acylhydrolases (E.C.3.1.1.3) (Joseph et al., 2008). Lipases are water-soluble enzymes which play a key role in fat metabolism and digestion (Reis et al., 2009). Lipases break and modify the carboxyl esters bonds of lipids and their derivates at the water-lipid interface and reversing the reaction in non-aqueous media (Joseph et al., 2008; Saxena et al., 2003). Hydrolysis of fat is the primary reaction of lipases (Joseph et al., 2008). Lipases catalyse a variety of reactions, such as partial or complete hydrolysis of

triaglycerols and reactions of esterification, transterification and interesterification, acidolysis, alcoholysis and aminolysis, in addition to the hydrolytic activity on triglycerides (Joseph et al., 2008; Salihu et al., 2012). The recent interest in the production of lipases is associated with their applications as additives in food industry, fine chemicals, detergent, wastewater treatment, cosmetics, pharmaceuticals, leather processing and biomedical assays (Salihu et al., 2012). Lipases have been successfully established for the synthesis of biopolymers, the production of enantiopure pharmaceuticals, agrochemicals, and flavour compounds (Hasan et al., 2006). In addition, lipases also have important application in the field of bioenergy, especially in biodiesel production (Salihu et al., 2012). Lipases are valued biocatalysts, since they act under mild conditions, are highly stable in organic solvents, show broad substrate specificity, and usually show high regio-and/or stereoselectivity in catalysis (Hasan et al., 2006).

The commercial use of lipases is a billion dollar business that comprises a wide variety of different applications. A global market for industrial enzymes was estimated in two billons dollars in 2004 (Joseph et al., 2008). Following proteases and carbohydrases, lipases are considered the third largest group, based on total sales volume. The majority of enzymes with industrial use are from microbial origin and are produced in conventional aerobic SmF (Hasan et al., 2006).

2.3.1 Lipase production by filamentous fungi

Numerous species of bacteria, yeasts and filamentous fungi produce lipases (Salihu et al., 2012). Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly due to the versatility of their applied properties and ease of mass production. Lipases isolated from different sources have a wild range of properties depending on their sources, with respect to positional specificity, fatty acid specificity, thermostability, optimum pH, etc. (Hasan et al., 2006). Filamentous fungi are recognised as the best lipase producers and are currently the preferred sources, since they produce extracellular lipases, facilitating the extraction from fermentation media (Contesini et al., 2010). Moreover, the hyphal mode of fungal growth and their good tolerance to low water activity make fungi extremely efficient in the bioconversion of the solid substrate (Díaz et al., 2007). The most reported species belong to the genera *Rhizopus*, *Mucor*, *Geotrichum*, *Penicillium*, and *Aspergillus* (Contesini et al., 2010). Table 2.8 presents some filamentous fungi producers of lipases.

Table 2.8 – Filamentous fungi producers of lipases.

Filamentous fungi	References
Aspergillus awamori	Basheer et al. (2011)
Aspergillus carbonarius	Drobev et al. (2015)
Aspergillus fumigatus	Coca et al. (2001)
Aspergillus ibericus	Abrunhosa et al. (2013), Oliveira et al. (2016), Salgado et al. (2014b)
Aspergillus niger	Coca et al. (2001), Damaso et al. (2008) Dutra et al. (2008) Falony et al. (2006), Edwinoliver et al. (2010), Ilmi et al. (2017), Mahadik et al. (2002), Mala et al. (2007), Kamini et al. (1998), Oliveira et al. (2016), Salgado et al. (2014b), Silveira et al. (2016), Venkatesagowda et al. (2015)
Aspergillus oryzae	Toida et al. (1998)
Aspergillus tubingensis	Oliveira et al. (2016)
Aspergillus uvarum	Salgado et al. (2014b)
Botrytis cinerea	Commenil et al. (1995)
Chalaropsis thielavioides	Venkatesagowda et al. (2015)
Colletotrichum gloeosporioides	Balaji and Ebenezer (2008), Venkatesagowda et al. (2015)
Fusarium solani	Knight et al. (2000)
Geotrichum candidum	Jacobsen and Poulsen (1995), Sidebottom et al. (1991)
Lasiodiplodia theobromae	Venkatesagowda et al. (2015)
Mucor hiemalis	Molla et al. (2004)
Mucor hiemalis f. *corticola*	Ülker and Karaoglu (2012)
Penicillium aurantiogriseum	Lima et al. (2004, 2003)
Penicillium candidum	Ruiz et al. (2001)
Penicillium chrysogenum	Kumar et al. (2011)
Penicillium citrinum	Miranda et al. (2000), Pimentel et al. (1997)
Penicillium fellutanum	Amin and Bhatti (2014)
Penicillium restrictum	Freire et al. (1997)
Penicillium simplicissimum	Gutarra et al. (2009)
Phanerochaete chrysosporium	Molla et al. (2004)
Phoma glomerata	Venkatesagowda et al. (2015)
Rhizomucor miehei	Herrgard et al. (2000), Ilmi et al. (2017)
Rhizopus arrhizus	Rajendran and Thangavelu (2009)
Rhizopus chinensis	Sun and Xu (2009, 2008), Wang et al. (2008)
Rhizopus homothallicus	Rodriguez et al. (2006)
Rhizopus oryzae	Ben Salah et al. (2007)
Talaromyces thermophilus	Romdhane et al. (2010)
Thermomyces lanuginosus	Ávila-Cisneros et al. (2014)
Trichoderma harzianum	Coradi et al. (2013), Molla et al. (2004)

2.3.2 *Aspergillus ibericus*

Aspergillus ibericus, eukaryotic, aerobic microorganism, belongs to the Fungi Kingdom, Ascomycota division, Eurotiomycetes class, Eurotiales order and Trichocomaceae family. This filamentous fungus is a black *Aspergillus* species within the section *Nigri*, which was isolated from wine grapes from Évora, Alentejo, Portugal, and etymology *"ibericus"* from Iberian Peninsula (Serra et al., 2006). Strains of *A. ibericus* do not produce detectable ochratoxin A (OTA) in laboratory media, presenting an potential advantage for its application in biotechnology (Serra et al., 2006). In grapes, the species responsible for OTA production is *A. carbonarius*. In Portuguese vineyards *A. ibericus* strains are less frequently isolated compared with *A. niger* aggregate and *A. carbonarius* isolates (Serra et al., 2006).

The black *aspergilli* have a relevant importance in biotechnology, since the most recognised species, *Aspergillus niger*, has the GRAS status from Food and Drug Administration (FDA). It has been demonstrated that *A. ibericus* produces lipase on olive mill wastewaters using SmF (Abrunhosa et al., 2013); on OP and winery wastes using SSF (Salgado et al., 2014b); and recently on OP with WB also by SSF (Oliveira et al., 2016), revealing the potential of this species to produce lipase by SSF using OP.

2.4 Factors affecting SSF for lipase production

2.4.1 Optimisation of the bioprocess

The selection of the microorganism, the substrate and the optimum process parameters must be considered and optimised in SSF (Singhania et al., 2009). Many studies have been undertaken to define the optimal culture conditions and nutritional requirements for metabolites production (Contesini et al., 2010).

Biochemical and physicochemical parameters such as the substrate to be used, its particle size, initial moisture content (MC), initial pH and pre-treatment of the substrate, supplementation with nutrients such as nitrogen, phosphorus, and trace elements, supplementation with additional carbon sources and inducers, age and size of the inoculum, and also the temperature of incubation (Pandey, 2003), have to be optimised in order to achieve higher metabolite productivities (Singhania et al., 2009). Among several critical parameters, MC and nature of solid substrate

employed are the most important parameters affecting SSF process. Selection of MC depends on microorganism employed and also the nature of substrate (Singhania et al., 2009). Fungi needs lower MC, being 40 - 60% MC required for SSF. Selection of substrate depends upon several factors mainly related with cost and availability, and thus may involve screening of several agro-industrial residues (Singhania et al., 2009).

In literature, some authors have compared SSF and SmF systems for lipase production. Colla et al. (2010) reported higher lipase productivities under SSF comparing SmF, using *Aspergillus* sp. on WB with soybean oil, obtaining 25.2 U and 4.5 U, respectively. Silveira et al. (2016) obtained higher lipase production by SSF comparing to SmF, using *Aspergillus niger* on palm oil wastes, yielding 15 U mL^{-1} and 10 U mL^{-1}, respectively. Several factors may affect extracellular lipase production. In addition, the presence of triglycerides or fatty acids has been reported to increase lipolytic enzyme secretion by a certain number of microorganisms. Therefore, in SSF the type of substrate could be used to enhance the production of enzymes, as several food and agro-industrial residues are rich in fatty acids, triglycerides and/or sugars (Couto and Sanromán, 2006).

The use of agro-industrial residues as substrate may result in a reduction in the costs of metabolites production, as enzymes, considering that the culture medium usually represents 25 - 50% of the total production costs (Contesini et al., 2010). Castilho et al. (2000) performed a comparative economic analysis of SSF and SmF processes for the production of lipase by *Penicillium restrictum*. They found for a plant producing of 100 m^3 lipase concentrate per year, the process based on SmF needed a total capital investment 78% higher than the based on SSF and its product had a unitary cost 68% higher than the product market price. These results showed the great advantage of SSF associated to the low cost.

2.4.2 Scale-up to industrial level

The main parameters to be measured and controlled in SSF processes are temperature, homogenous aeration, pH, and MC (Bellon-Maurel et al., 2003). All this factors affect the reactor design and the control strategy for the parameters. In SmF the main limiting one is oxygen transfer. Whereas, in SSF, besides this, and depending on the bioreactor design, two other parameters are of crucial importance: the temperature and the MC of the substrate. Other factors affecting the

bioreactor design are the morphology of the fungus, its resistance to mechanical agitation (in case of rotary-drum) and the need or not to have a sterile process. In general, several types of bioreactors are able to run at laboratory scale with small amount of substrate, but scale-up may be complicated by the intense heat generation and the heterogeneity in the system (Couto and Sanromán, 2006).

Commonly bioreactors used in SSF includes packed-bed, tray-type and horizontal rotary-drum, having their own advantages and disadvantages, which promotes the necessity to develop novel bioreactors (Singhania et al., 2009). Pressurised SSF can solve some problems, since it may enhance mass and heat transfer of the process (Xu et al., 2002).

A packed-bed bioreactor typically involves a static bed on top of a perforated plate through which conditioned air is blown. Due to the end-to-end aeration, axial temperature gradients are impossible to prevent (Mitchell et al., 2000). Gutarra et al. (2005) found that the temperature in the inner bed substrate was higher than the temperature set in the water jacket of the packed-bed bioreactor. These axial temperature gradients promote evaporation even using saturated air, since the water carrying capacity of the air increases as it heats up (Mitchell et al., 2000). A strategy for minimising axial temperature gradients in packed-bed bioreactors is suggested by Lu et al. (1998) who divided the bed into layers, creating a multi-layer packed-bed bioreactor. This led to improve heat and mass transfer in comparison to a single packed-bed bioreactor.

A tray-type bioreactor consists in a chamber in which air, with controlled temperature and relative humidity, circulates around a number of trays. Each tray contains a thin layer of substrate, and usually has an open top and a perforated bottom; also, the relative humidity should be high avoiding the drying of the bed surface (Mitchell et al., 2000). As the substrate bed height increases, the mass and energy transfer become more difficult. For instance, the metabolic heat produced during the fermentation process accumulates in the substrate bed, creating a temperature gradient (Vaseghi et al., 2013). Furthermore, the mycelium growth during the fermentation period accumulates in the substrate bed, and hinders the diffusion of air inside all the substrate (Vaseghi et al., 2013). Rajagopalan and Modak (1994) reported that the scale-up may be achieved by increasing the area of trays, using wider trays, or simply using more trays. Figueroa-Montero et al. (2011) considered two main variables in SSF in tray-type bioreactor, the substrate bed height and the appropriate air supply needed for the aerobic process and for heat dissipation, in order to avoid/or decrease the drying in the substrate bed.

Horizontal rotary-drum bioreactor typically consists in cylindrical drum, where the agitation is applied by a rotating system around the central axis, or by paddles within the container (Bellon-Maurel et al., 2003). A SSF is initiated filling the bioreactor with inoculated substrate, occupying 10% to 40% of the bioreactor volume (Hardin et al., 2000). It is assumed a homogeneity in headspace gases and in thermal and moisture equilibrium with the substrate bed, which is also well mixed (Hardin et al., 2000). The drum is rotated continuously at a slow speed to mix the substrate bed. In practice, rotation rates of 2 to 3 rpm are most common, although values as low as 3 revolutions per day and as high as 40 to 50 rpm have been reported (Hardin et al., 2000). The effect of rotational speed on SSF, particularly, the effect of shear caused by drum rotation is controversial. In some studies, high rotation reduces SSF productivity and sporulation, due to the deleterious effects of shear; however, in other studies productivity was higher at highest rotational speed, promoting higher aeration and microorganism growth, but this led to an overheating of the system, and heat removal was a key issue (Stuart et al., 1999).

2.5 Conclusions

In the reported literature, SSF offers several potential advantages for bioprocessing and production of various added-value compounds. In many cases, the metabolite productivities achieved in SSF are higher than in SmF. The use of agro-industrial residues as substrate for bioconversion to obtain a metabolite is another important factor, becoming a cheaper process. Between various parameters, the choice of substrate may allow metabolite yield increase, as for example, the lipase production using OCs as substrates, since it contains residual oil. Several technical challenges have been pointed especially with regard to improvements using bioreactors for large scale production.

3 Influence of moisture content and temperature on lipase produced by *Aspergillus* species

Pollution by olive mill wastes is an important problem in the Mediterranean area and novel solutions for their proper management and valorisation are needed. The aim of this work was to optimise a solid-state fermentation (SSF) process to produce lipase using olive pomace (OP) as the main source of nutrients by several *Aspergillus* spp. Optimised variables were moisture content (MC) and temperature.

Results showed that the MC was the most significant factor affecting lipase production for all fungi strains tested. With MC and temperature optimisation, a 4.4-fold increase in *A. ibericus* lipase was achieved (90.5 ± 1.5 U g^{-1}), using a mixture of OP and wheat bran (WB) at 1:1 ratio, 0.02 g $NaNO_3$ per g dry substrate, 60% of MC and incubation at 30 °C for 7 days. For *A. niger* and *A. tubingensis*, highest lipase activity obtained was 56.6 ± 5.4 U g^{-1} and 7.6 ± 0.6 U g^{-1}, respectively.

A. ibericus was found to be the most promising microorganism for lipase production using mixtures of OP and WB.

The information presented in this chapter was published and presented, as follows:

Oliveira, F., Moreira, C., Salgado, J. M., Abrunhosa, L., Venâncio, A., & Belo, I. (2016). Olive pomace valorization by *Aspergillus* species: lipase production using solid-state fermentation. *Journal of the Science of Food and Agriculture*, 96, 3583–3589.

Oliveira, F., Salgado, J. M., Abrunhosa, L., Venâncio, A., & Belo, I. (2013). Influence of moisture content, temperature and inoculum size on lipase production by filamentous fungi under solid-state fermentation of olive pomace. In *Book of Abstracts of MicroBiotec´13 – Portuguese Congress of Microbiology and Biotechnology*, pp. 124. Aveiro, Portugal. 6-8 December.

3.1 Introduction

Olive pomace (OP) is a sludgy waste generated by the olive oil two-phase extraction system. The two-phase extraction system comes to be largely implemented in new olive mills, turning OP the most important residue produced. OP is an acidic and very humid solid by-product, that is rich in organic matter, carbohydrates, phenols and also contains residual fats (Alburquerque et al., 2006b). Its properties make it an interesting substrate to induce lipase production by filamentous fungi under solid-state fermentation (SSF). Recently, a growing interest in lipase production in low-cost agro-industrial residues has emerged, and the use of substrates such as wheat bran (WB) has been proposed (Damaso et al., 2008; Falony et al., 2006). The access to starch fraction and high protein concentration add an extra value to WB, compared to other lignocellulosic feedstocks (Apprich et al., 2014).

In herein reported work, OP was mixed with WB. The aim of the work was to optimise the production of lipase by *Aspergillus ibericus* MUM 03.49, *Aspergillus niger* MUM 03.58 and *Aspergillus tubingensis* MUM 06.152, under SSF. The optimisation of the process was performed evaluating parameters such as moisture content (MC) and temperature.

3.2 Materials and methods

3.2.1 Microorganisms

Aspergillus ibericus MUM 03.49, *Aspergillus niger* MUM 03.58 and *Aspergillus tubingensis* MUM 06.152 (MUM culture collection, Braga, Portugal) were used. These strains demonstrated high lipolytic activity in previous work (Salgado et al., 2014b). They were revived on malt extract agar (MEA) plates (20 g L^{-1} malt extract, 20 g L^{-1} glucose, 1 g L^{-1} peptone and 20 g L^{-1} agar) at 25 °C from a frozen glycerol stock. Spore suspensions of the inoculum were prepared from seven-day-old culture plates with 1 g L^{-1} peptone solution (with 0.1 g L^{-1} Tween 80). The spore concentration of the suspension was adjusted to 10^6 spores mL^{-1}.

3.2.2 Substrates

OP samples were collected from a two-phase olive mill plant, during the campaign 2011/2012, in Vila Real, Portugal, and stored at -20 °C. WB was purchased in a local supermarket. OP characteristics were determined by Salgado et al. (2014a) and are presented in Table 3.1. The particle size of OP was less than 1 mm. The organic constituents of OP such as cellulose, hemicellulose and Klason lignin were characterised by quantitative acid hydrolysis (QAH) in a two-stage acid treatment. The first stage with 72% wt H_2SO_4 at 30 °C for 1 h and the second stage after dilution to 4% wt H_2SO_4 at 121 °C for 1 h. The solution was filtered through a Gooch crucible to retain lignin and these were introduced in the hot air oven at 105 °C. The filtrate was analysed by High Performance Liquid Chromatography system using a Jasco830-IR intelligent refractive-index detector and a Varian MetaCarb 87H column. The column was eluted with 0.005M H_2SO_4 and the flux was 0.7 mL min^{-1} at 60 °C (Leite et al., 2016).

Table 3.1 – Characteristics of olive pomace (OP). Values are the mean of triplicate analysis ± standard deviation (SD) (Salgado et al., 2014a).

Characteristics of OP	Value ± SD
MC (%, w/w)	75.3 ± 0.1
Total solids (%, w/w)	24.7 ± 0.1
Lignin (g kg^{-1})	582 ± 4
Hemicellulose (g kg^{-1})	42 ± 2
Cellulose (g kg^{-1})	68 ± 2
Lipids (g kg^{-1})	102 ± 0.4
Protein (g kg^{-1})	0.30 ± 0.03
Reducing sugars (g kg^{-1})	24 ± 1
Total phenols (g kg^{-1})	2.57 ± 0.04
N (%, w/w)	0.8 ± 0.2
C (%, w/w)	51.7 ± 1.5
C/N ratio	60 ± 8

Mass per total dry mass of OP, exception for MC

Nitrogen and carbon in solid residues were analysed using a Thermo Finningan Flash Elemental Analyzer 1112 series, San Jose CA (USA). To determine free reducing sugars, total phenols, and proteins in solid residues, extraction with water 1:5 (w/v) was performed. Reducing

sugars were determined by dinitrosalicylic acid method (Miller, 1959). Lipids (total fat content) were extracted with diethyl ether in a Soxtec System HT2 1045 extraction unit. Protein was determined by Bradford method (Bradford, 1976). Total phenols were determined by the Folin-Ciocalteu method using caffeic acid as a standard. Total solids were analysed by oven drying to a constant weight at 105 °C (Salgado et al., 2014a).

3.2.3 SSF experiments

SSFs were performed in cotton-plugged 500 mL Erlenmeyer flasks containing 30 g dry solid substrate (Figure 3.1). Initial MC was adjusted with distilled water when necessary. Flasks were prepared, autoclaved at 121 °C for 15 min, cooled, inoculated with 1 mL of spores suspension and incubated at different temperatures and fermentation times. Optimum SSF conditions for each fungus were determined in previous work, through Taguchi L9 orthogonal array (Table 3.2), and were used as the starting point for these SSF experiments (Oliveira et al., 2016).

Figure 3.1 – Erlenmeyer´s flask containing fermented substrate.

Table 3.2 – Optimum SSF conditions used for each fungus (optimisation with Taguchi L9 orthogonal array) (Oliveira et al., 2016).

Fungus	OP:WB mass ratio	$NaNO_3$ (g)	Czapek nutrients	Time (d)
A. ibericus	1:1	0.6	0	7
A. niger	2:1	0.15	0	14
A. tubingensis	2:1	0.15	0	21

OP – olive pomace, WB – wheat bran

3.2.4 Optimisation of SSF with full factorial design

A full factorial design (3^2) was performed using Statistica 12 software (StatSoft, Tulsa, USA) in order to study the influence of MC and temperature on the production of lipase. As shown in Table 3.3, three levels were assigned to each factor: MC (70, 75 and 80%) and temperature (25, 30 and 35 °C), performing 9 runs total. Polynomial equations were fitted to experimental values of lipase activity using Statistica 12 software and best levels of MC and temperature that originated the maximum lipase activity were determined using the Solver application from Microsoft Excel 2010. The relationship between the dependent (lipase activity) and independent (MC and temperature) variables was established by the polynomial Eq. (3.1), as follows:

$$y = a + a_1 x_1 + a_2 x_2 + a_{11} x_1^2 + a_{22} x_2^2 + a_{12} x_1 x_2 \quad (3.1)$$

Where y is the predicted response, x_1 and x_2 are the independent variables, a is the intercept, a_1 and a_2 are the linear coefficients, a_{11} and a_{22} are the quadratic coefficients and a_{12} is the interaction coefficient.

3.2.5 Optimisation of SSF at low levels of MC

For each fungus at optimum temperature found, an optimisation of MC was conducted at levels between 35% and 70%, performing 8 runs total.

3.2.6 Lipase extraction and determination

At the end of the incubation period, enzyme was extracted by adding 150 mL of 10 g L^{-1} NaCl and 5 g L^{-1} Triton X-100 (5 mL g^{-1} dry solid substrate) to the fermented substrates and mixed at 170 rpm and 20 °C for 2 h using a shaker. Mixtures were then centrifuged (12000 × g and 10 min at 4 °C) and filtered using a Whatman N° 1 filter paper. The resulting enzymatic extracts were immediately used for lipase determination.

Lipase activity was determined by a spectrophotometric method, using *p*-nitrophenyl butyrate (pNPB) as described by Gomes et al. (2011). One unit of lipase activity (U) was expressed as the amount of enzyme which produces 1 µmol of *p*-nitrophenol (pNP) per minute, under the assay conditions. The analyses were performed in triplicate. Lipase activity obtained was expressed as units per gram of dry solid substrate (U g^{-1}).

3.2.7 Statistical treatment

Data obtained were statistically analysed using SPSS (IBM SPSS Statistics, Version 22.0. Armonk, NY: IBM Corp.) to study the effect of variables on lipase production. Data were tested for homogeneity, submitted to one-way analysis of variance (ANOVA) and a pair-wise multiple comparison procedure (Tukey test) at a confidence level of 95%.

3.3 Results and discussion

3.3.1 Optimisation with full factorial design

Table 3.3 presents experimental values of lipase activity from the full factorial design for the filamentous fungi tested, as a function of MC and temperature. *A. ibericus* and *A. niger* reached the highest lipase activity (28.0 ± 1.0 U g^{-1} and 16.6 ± 0.5 U g^{-1}, respectively) at 70% MC and 30 °C. For *A. tubingensis*, a maximum of 6.7 ± 0.6 U g^{-1} was obtained at 70% MC and 25 °C. Compared to the previous results from optimisation conditions using Taguchi design, these results corresponds to an increase in lipase production of 35% for *A. ibericus*, 64% for *A. niger* and 14% for *A. tubingensis*, by MC and temperature optimisation. The effects of those factors on lipase production were determined using Statistica software and it was observed that an increase of MC from 70% to 80% had a negative effect ($p < 0.005$) on lipase production by *A. ibericus* and *A. niger*, but was insignificant ($p > 0.05$) for *A. tubingensis*. Concerning temperature, a significant effect on lipase production was only observed for *A. ibericus* ($p < 0.05$).

In other works, MC was also considered one of the most important factors affecting SSF processes (Singhania et al., 2009), as well as temperature. Maximum saturation of enzyme active site occurs at optimum temperature, as enzyme gets denatured at high temperature. Also, at high

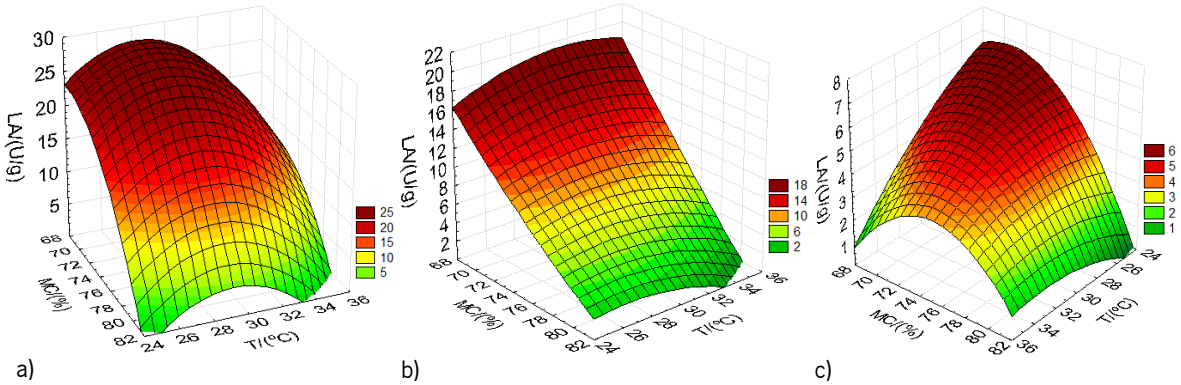
temperature, protease production occurred that denatures the enzymes (Kumar et al., 2011). However, at lower temperature, the microorganism´s growth slows down and consequently the enzyme production.

Table 3.3 – Factors and assigned levels of full factorial design. Experimental and predicted values of lipase activity (LA) obtained for each fungus. Experimental LA values are the mean of triplicate analysis ± standard deviation (SD).

			Experimental and predicted LA ± SD (U g^{-1})					
			A. ibericus		*A. niger*		*A. tubingensis*	
Run	MC (%)	Temp. (°C)	Experim.	Predict.	Experim.	Predict.	Experim.	Predict.
1	70	25	23.2 ± 5.2	24.9	13.9 ± 1.2	14.4	6.7 ± 0.6	6.5
2	70	30	28.0 ± 0.6	27.2	16.6 ± 0.5	16.1	5.2 ± 0.6	5.0
3	70	35	16.7 ± 1.1	16.4	15.8 ± 0.8	15.3	2.6 ± 0.6	2.6
4	75	25	20.8 ± 1.0	19.3	10.1 ± 0.7	8.8	5.9 ± 1.1	5.6
5	75	30	22.9 ± 1.5	22.4	9.6 ± 0.9	9.5	4.8 ± 1.0	5.2
6	75	35	9.8 ± 0.6	12.4	6.9 ± 0.4	7.7	4.3 ± 1.1	3.8
7	80	25	6.8 ± 1.1	7.1	3.6 ± 0.8	3.9	1.9 ± 0.3	2.0
8	80	30	9.0 ± 0.5	10.9	3.7 ± 0.5	3.7	3.3 ± 0.2	2.7
9	80	35	3.2 ± 0.2	1.6	1.7 ± 0.4	0.9	2.2 ± 0.2	2.4

Following, polynomial equations as a function of moisture content (MC) and temperature (T) were fitted to the experimental values of lipase activity (LA) for each fungus (Figure 3.2). The analysis of variance indicates a good fitting of the equations to the experimental values ($R^2 > 0.96$), with the exception for *A. tubingensis* ($R^2 < 0.80$). According with the model equations, the variation of lipase activity with MC and temperature were represented as response surface plots, using Statistica software (Figure 3.2). There, the negative effect on lipase activity of increasing MC levels and the existence of optimum values of MC and temperature, for which, lipase activity is maximised, using Solver application, can be clearly observed (Figure 3.2 and Table 3.4).

In general, results revealed that optimum values of temperature were around 30 °C for *A. ibericus* and *A. niger,* and 25 °C for *A. tubingensis*, but that the optimum MC was close to the lower level of the experimental design for all strains (around 70%). Thus, further experiments with MC lower than 70% were conducted to find the optimum.



	Fungus	Equation
a)	*A. ibericus*	$LA = -753.07 + 17.52MC + 12.67T - 0.13MC^2 - 0.26T^2 + 0.03MCT$
b)	*A. niger*	$LA = 55.92 - 2.31MC + 5.91T + 0.015MC^2 - 0.05T^2 - 0.04MCT$
c)	*A. tubingensis*	$LA = -197.58 + 6.60MC - 2.22T - 0.05MC^2 - 0.02T^2 + 0.04MCT$

Figure 3.2 – Response surfaces of lipase activity (LA) as a function of moisture content (MC) and temperature (T) according to the polynomial equations of a) *A. ibericus* MUM 03.49, b) *A. niger* MUM 03.58 and c) *A. tubingensis* MUM 06.152.

Table 3.4 – Analysis of variance (ANOVA) of the polynomial equations and optimum predicted conditions of moisture content (MC) and temperature (T), and respective lipase activity (LA) for each fungus.

Analysis of variance (ANOVA)						
Fungus	R^2	R^2 adjusted	SS equation	SS residual	*F*-value	*p*-value
A. ibericus	0.9654	0.9308	580.16	20.80	27.89	0.0035
A. niger	0.9692	0.9383	236.58	7.53	31.44	0.0028
A. tubingensis	0.7603	0.5205	17.10	5.39	3.17	0.1449

	Optimum predicted		
Fungus	MC (%)	T (°C)	LA (U g^{-1})
A. ibericus	68.7	28.3	28.1
A. niger	70.0	30.9	16.2
A. tubingensis	70.9	25.0	6.6

R^2 – coefficient of determination, SS – sum of squares

3.3.2 Optimisation of SSF at low levels of MC

In order to study the effect of MC at lower values than the ones used in the previous experimental design, SSF experiments were carried out at optimal temperature obtained for the 3

fungi strains and at a wide range of MC values below 70%. Figure 3.3 presents results of lipase activity as a function of MC for each fungus. Lipase production was significantly higher for all strains at MC values lower than 70% ($p < 0.0001$) and a maximum was obtained at 60%, 50% and 35% for *A. ibericus*, *A. niger* and *A. tubingensis*, respectively. Comparing with MC at 70%, a 3-fold increase in lipase production was observed for *A. ibericus*, a 7-fold for *A. niger* and a 4-fold for *A. tubingensis*, yielding significantly higher lipase production. Again, *A. ibericus* produced more lipase (90.5 ± 1.5 U g^{-1}) than *A. niger* (56.6 ± 5.4 U g^{-1}) and *A. tubingensis* (7.6 ± 0.6 U g^{-1}). Comparing to *A. ibericus* lipase production from Taguchi design (at 75% MC and at 25 °C), 20.8 ± 1.0 U g^{-1}, a 4.4-fold increase was found.

The observed decrease of lipase activity with increasing MC has been observed by other researchers as well (Imandi et al., 2013; Lu et al., 2003; Pal and Khanum, 2010; Sun and Xu, 2008), and have been attributed to the impact of moisture on the physical properties of the solid substrate (Virupakshi et al., 2005). High MC decreases substrate porosity, alters substrate particle structure, promotes development of stickiness, reduces gas volume and exchange, leading to oxygen diffusion limitation in the substrate layer (Edwinoliver et al., 2010; Hamidi-Esfahani et al., 2004; Singhania et al., 2009; Sun and Xu, 2008), and microbial growth decreases (Hamidi-Esfahani et al., 2004). This study revealed the great importance of performing SSF at optimum MC to improve lipase production.

In general, after the optimisation process, *A. ibericus* was the best lipase producer, and a final increase of 4.4-fold in lipase production was achieved as a result of MC and temperature optimisation. The obtained maximum lipase activities (90.5 ± 1.5 U g^{-1} for *A. ibericus* and 56.6 ± 5.4 U g^{-1} for *A. niger*) were higher than those obtained by other researchers, which also used SSF and filamentous fungi to produce lipase. For example, Falony et al. (2006) reported a lipase activity of 9.1 U g^{-1} at 65% MC with *A. niger* using WB with olive oil as inductor; Guatarra et al. (2009) obtained a lipase production of 19.6 U g^{-1} in 72 h at 30 °C growing *Penicillium simplicissimum* in babassu cake supplemented with sugar cane molasses at 70% MC; and Sun and Xu (2008) reported the production of 24.5 U g^{-1} by *Rhizopus chinensis* in wheat flour with WB at 70% MC.

As it was mentioned in other work, *A. ibericus* is also capable of producing lipase under SmF using other olive residue, olive mill wastewater (OMWW). The maximum concentration of lipase activity obtained in that case was 8.3 U mL^{-1} (Abrunhosa et al., 2013), which was approximately 1.9-fold less than the one obtained in present work via SSF of OP with WB using *A. ibericus* (15.6

U mL^{-1} in the extracting solution). These results agree with other researchers, which also reported that SSF indeed leads to higher lipase concentration than SmF (Colla et al., 2010; Falony et al., 2006). Additionally, for *A. ibericus*, the observed optimum MC (60%) turned out to be very convenient because it is exactly the MC obtained when OP and WB are mixed at a ratio of 1:1, thereby contributing to the reduction of costs and simplification of an eventual process at a large scale.

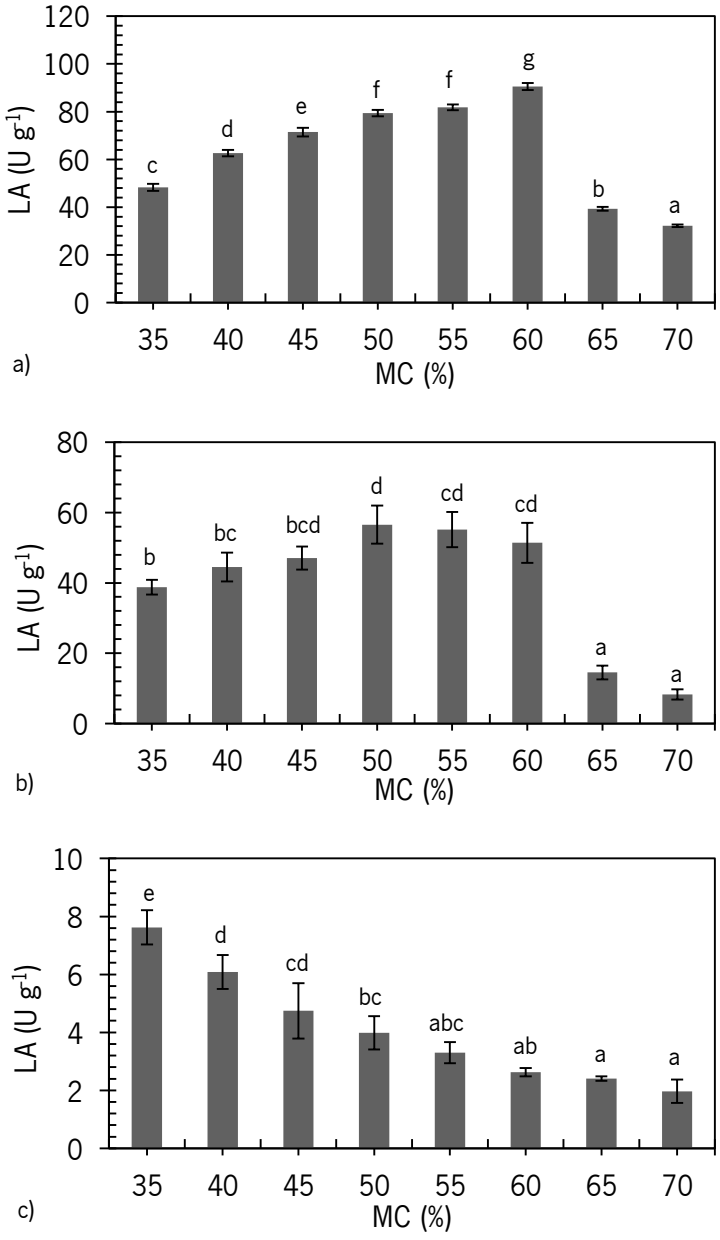


Figure 3.3 – Influence of moisture content (MC) on lipase activity (LA) of a) *A. ibericus* MUM 03.49, b) *A. niger* MUM 03.58 and c) *A. tubingensis* MUM 06.152. Depicted values are the mean of triplicate analysis ± standard deviation. Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

3.4 Conclusions

In conclusion, MC was found to be the most critical factor for lipase production by SSF with *Aspergillus* spp. Through optimisation of MC and temperature it was achieved an *A. ibericus* lipase production of 90.5 ± 1.5 U g^{-1}, which corresponds to 4.4-fold increase. The optimum SSF conditions were 60% of MC and incubation at 30 °C. *A. ibericus* was found to be the best lipase producer among tested strains, being a promising microorganism for the production of this enzyme under SSF of OP with WB, and enables an interesting approach for OP valorisation.

4 Optimisation of lipase production by solid-state fermentation of olive pomace: from flask to lab-scale packed-bed bioreactor

Lipases are versatile catalysts with many applications and can be produced by solid-state fermentation (SSF) using agro-industrial residues. The aim of this work was to maximise the production of *A. ibericus* lipase under SSF of olive pomace (OP) and wheat bran (WB), evaluating the effect of C/N ratio, lipids, phenols and sugars content of substrates on lipase production, and nitrogen source addition. Moreover, the implementation of the SSF process in a packed-bed bioreactor, and the lipase extraction conditions were assessed.

Low C/N ratios and high lipids content of the substrate led to maximum lipase production. Optimum SSF conditions were achieved with a C/N mass ratio of 25.2 and 10.2% (w/w) lipids, by the mixture of OP:WB (1:1) and supplemented with 1.33% (w/w) $(NH_4)_2SO_4$. Studies in a packed-bed bioreactor showed that aeration rate is an important factor, and that low rates were suitable for lipase production. In this work, the important role of Triton X-100 on lipase extraction from the fermented solid substrate was shown. A final lipase activity of 223 ± 5 U g^{-1} (dry basis) was obtained, after 7 days of fermentation. Optimisation of substrate composition allowed to increase lipase production using a low cost process.

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Oliveira, F., Abrunhosa, L., Venâncio, A., Belo, I., Pérez-Rodríguez, N., & Domínguez, J. M. (2015). *Aspergillus ibericus* lipase production by solid-state fermentation of olive pomace. In C. Vilarinho, F. Castro, & M. Russo (Eds.), *Wastes: Solutions, Treatments and Opportunities* (Taylor & Francis Group), pp. 195–201. London. ISBN: 978-1-138-02882-1. (Selected papers from the 3rd edition of the International Conferences on Wastes: Solutions, Treatments and Opportunities, Viana do Castelo, Portugal, 14-16 September).

4.1 Introduction

Many enzymes manufacturers use submerged fermentation (SmF) techniques for enzymes production (Viniegra-González et al., 2003). However, in recent years, solid-state fermentation (SSF) was shown to be very promising for enzymes and other bioproducts production at very interesting concentrations (Pandey et al., 2000). SSF is considered a low cost raw material process due to the use of agro-industrial residues, thus SSF adds an economic advantage in enzymes production (Singhania et al., 2009). The selection of suitable solid substrate composition is a key factor in SSF. Several aspects should be taken into account, such as source of C and N, inducers of enzymes expression, costs and availability of substrates, particle size and moisture content (MC) (Pandey et al., 2000).

The evaluation of lab-scale bioreactor is a necessary step before carrying out the industrial scale-up. A packed-bed bioreactor is one of bioreactor type suitable for SSF. It typically involves a static bed on top of a perforated plate through which conditioned air is blown. These bioreactors have several advantages as easy to handle, can operate in continuous and the extraction of enzymes can be performed in situ (Ganguly and Nandi, 2015).

Thus, with the aim of improving the production of lipase by *Aspergillus ibericus* MUM 03.49 using SSF of olive pomace (OP) with wheat bran (WB), several variables affecting lipase secretion were studied, such as source of nitrogen and composition of mixtures of OP and WB. Under the best conditions obtained, the process was implemented in a packed-bed bioreactor and the effect of aeration rate was assessed. Additionally, lipase extraction conditions were optimised.

4.2 Materials and methods

4.2.1 Microorganism

Aspergillus ibericus MUM 03.49 was used, as described in chapter 3.2.1, but it was grown at 30 °C. And, the spores concentration of the suspension was adjusted to 10^7 spores mL^{-1}.

4.2.2 Substrates

OP from campaign 2013/2014 and WB were used, similarly to the described in chapter 3.2.2. Characteristics of OP were determined by Leite et al. (2016), as described in chapter 3.2.2 and in chapter 5.2.8. OP is very humid, with MC of 73.5 ± 0.4% (w/w). It contains reducing sugars in a concentration of 9.6 ± 0.6% (w/w), total phenols 0.84 ± 0.03% (w/w) and high C/N mass ratio of 82.8, indicating the need of nitrogen supplementation. OP also contains a considerable concentration of lipids (16.7 ± 0.1% (w/w)), which induces lipase production (Leite et al., 2016). WB characteristics were determined through the methodology described in chapter 3.2.2 and in chapter 5.2.8. WB presents a MC of 12.5 ± 0.1% (w/w), reducing sugars of 1.5 ± 0.2% (w/w), total phenols of 0.38 ± 0.03% (w/w), and a C/N ratio of 17.2. Complete characterisation of OP and WB is presented in Table 5.3, from chapter 5.3.5 (page 75).

4.2.3 Optimisation of lipase production by SSF

SSFs were performed in cotton-plugged 500 mL Erlenmeyer flasks containing 30 g dry solid substrate in a ratio of 1:1 (w/w, dry basis) of wet OP and WB. The mixture of OP with WB resulted in MC of around 60%, without the need of adjustment. Flasks were prepared, autoclaved at 121 °C for 15 min, cooled, inoculated with 1 mL of spores suspension and incubated at 30 °C during 7 days (SSF optimisation from chapter 3). After the incubation period the fermented substrates were extracted as described below to obtain the enzymatic extracts.

Different sets of SSF experiments were conducted to study the influence of the addition of different nitrogen sources and of substrates composition in the production of lipase by *A. ibericus*. Urea, $NaNO_3$, NH_4Cl and $(NH_4)_2SO_4$ were used as nitrogen source at a concentration of 2% (w/w) in dry solid substrate. $(NH_4)_2SO_4$ was further evaluated at concentrations that ranged from 0% to 3.33%, since it was the nitrogen source with the most pronounced effect in previous evaluation.

Different ratios of dried and ground OP:WB (OP:WB mass ratios of 1:0, 3:2, 1:1, 2:3, 1:4 and 0:1) and mixtures of olive oil (0, 1, 2.5, 5 and 10% (w/w)) with 30 g of dry solid WB were tested to evaluate the effect of different C/N ratios and % of lipids on lipase production. In this set of experiments, MC was adjusted to 60% (w/w).

After the optimisation of SSF conditions, a set of experiments was performed to monitor the time course of lipase production. Flasks were prepared as described before and destructively sampled each 2 days over a period of 20 days.

4.2.4 SSF in a packed-bed bioreactor

The horizontal packed-bed bioreactor used consisted in a double jacketed glass column (34 cm length and 3 cm internal diameter) connected to a filtered-air supply (Figure 4.1). The air was passed through a 0.45 μm cellulose filter and bubbled in distilled water before entering in the column. The air flow was measured and controlled by a flowmeter (Aalborg Instruments & Controls, Inc., USA). The bioreactor and 25 g of dry solid substrate were previously sterilised separately. Substrate was inoculated with 1 mL of inoculum suspension and mixed. The column was completely filled with the inoculated substrate, and incubated at 30 °C during 7 days. SSFs were performed at different aeration rates of 0.05 L min^{-1}, 0.1 L min^{-1} and 0.2 L min^{-1}. SSF experiments at bioreactor were performed in triplicate. Also, an additional SSF without aeration was performed. After SSF, the fermented substrate was removed from the column and transferred to a 500 mL Erlenmeyer flask to proceed with the enzyme extraction.

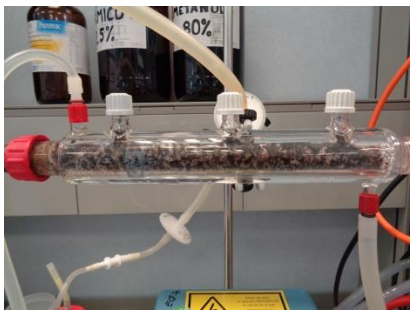

Figure 4.1 – Lab-scale packed-bed bioreactor containing fermented substrate.

4.2.5 Lipase extraction and determination

Lipase extraction was performed as described in chapter 3.2.6.

Lipase activity was determined by a spectrophotometric method, using a reaction mixture composed by 5 µL of enzymatic extract with 300 µL of 2 mM *p*-nitrophenyl butyrate (pNPB) in potassium phosphate 50 mM at pH 7.0. The absorbance was measured at 405 nm after reaction during 15 min at 37 °C. One unit of lipase activity (U) was expressed as the amount of enzyme which produces 1 µmol of *p*-nitrophenol (pNP) per minute, under the assay conditions. The analyses were performed in triplicate. Lipase activity obtained was expressed as units per gram of dry solid substrate (U g^{-1}).

4.2.6 Optimisation of lipase extraction

In order to assess if lipase extraction conditions above described in chapter 3.2.6 could be further optimised, variables such as type and volume of solvent were varied. Moreover, five consecutive extractions were performed in the same fermented substrate to determine the extraction recovery.

4.2.7 Statistical treatment

The multiple linear regression was carried out using Statgraphics Centurion XVI software (Statpoint Technologies, Inc. Warrenton, V).

Data obtained were statistically analysed using SPSS, as described in chapter 3.2.7.

4.3 Results and discussion

4.3.1 Optimisation of lipase production by SSF

4.3.1.1 Effect of nitrogen sources

In previous work, Oliveira et al. (2016) observed a positive influence of a nitrogen source, $NaNO_3$, on *A. ibericus* lipase. Here, the influence of different nitrogen sources on lipase production was studied. For all the nitrogen sources used, a significant positive effect ($p < 0.001$) on lipase production was obtained by the nitrogen source addition (Table 4.1), compared to the SSF without

nitrogen supplementation. Highest lipase activity was obtained using 2% (w/w) of ammonium salts, NH_4Cl and $(NH_4)_2SO_4$ with C/N ratio close to 23. The positive effect of $(NH_4)_2SO_4$ over urea was also observed in production of lipase by *Yarrowia lipolytica* in SSF of OP with WB (Lopes et al., 2016). On the contrary, other authors found urea as the best nitrogen source, in comparison to $(NH_4)_2SO_4$ and/or NH_4Cl, for the production of lipase by SSF using other fungi species and substrates (Balaji and Ebenezer, 2008; Imandi et al., 2010; Rodriguez et al., 2006).

Table 4.1 – Lipase activity (LA) affected by the different nitrogen sources at a concentration of 2% (w/w). LA values are the mean of triplicate analysis ± standard deviation (SD). Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

N source supplementation	Without N source	Urea	$NaNO_3$	NH_4Cl	$(NH_4)_2SO_4$
C/N mass ratio	29.7	18.8	24.7	22.4	23.5
LA ± SD (U g^{-1})	89 ± 5^a	106 ± 7^b	115 ± 6^b	144 ± 5^c	151 ± 7^c

Figure 4.2 presents lipase yields of experiments conducted with different concentrations of $(NH_4)_2SO_4$. The increase of $(NH_4)_2SO_4$ concentration above 2% did not improve lipase production but the decrease below 0.67% had a negative impact.

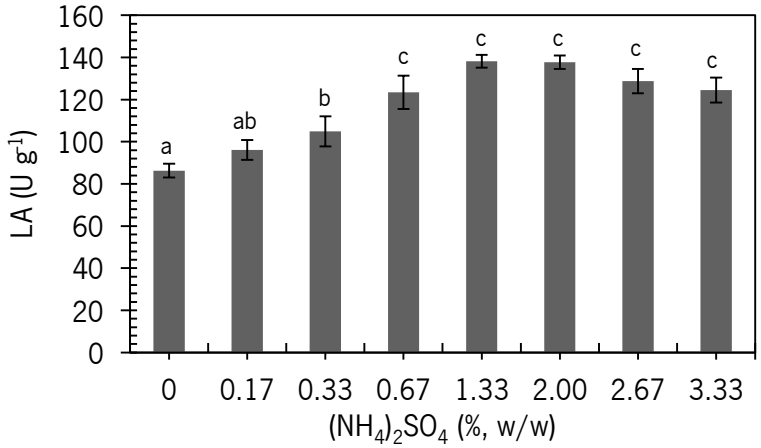

Figure 4.2 – Profile of lipase activity (LA) as a function of the amount of $(NH_4)_2SO_4$ added to the substrate. Depicted values are the mean of triplicate analysis ± standard deviation. Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

Different nitrogen sources and concentration levels have been used to improve lipase production under SSF. For example, Imandi et al. (2013) supplemented mustard oil cake with 1.5% (w/w) of urea to improve production of lipase by *Yarrowia lipolytica*, obtaining around 55 U g^{-1} of lipase. Whereas, Salgado et al. (2014b) observed the need of adding 7.3% (w/w) of exhausted grape mark (source of nitrogen) to OP to optimise the production of lipase by *A. ibericus*, yielding 14.8 U g^{-1}. In the present work, 1.33% (w/w) of $(NH_4)_2SO_4$ was selected to perform SSF in next experiments.

4.3.1.2 Effect of C/N ratio and lipids in lipase production

Mixtures of OP, WB and olive oil were studied to evaluate the effect of C/N ratio and the lipids content of substrate on lipase production. Table 4.2 shows the mixtures used, their C/N ratio, lipids, phenols and sugars content in the mixture, and the lipase activities achieved. As can be seen, low C/N ratios (22 - 25) led to high lipase activities. No lipase production was observed in SSF with C/N ratio of 56.5, and C/N ratios below the value of 22 reduced the lipase production. Often, the agro-industrial residues have a high C/N ratio due to their low content in N, as OP. Thus, these residues are supplemented with N source to carry out biotechnology processes, which increased the costs of process. This can be avoided using mixture of residues to adjust the C/N ratio, and by optimisation of this ratio so that the substrate is not needlessly over supplemented.

Jia et al. (2015) observed that this parameter had significantly influenced lipase production by *Aspergillus* sp. in SmF. However, its effect in SSF with *Aspergillus* sp. was not studied. Other works studied lower C/N ratios for production of lipases by SSF using *Penicillium* sp. Rigo et al. (2009) evaluated the range of C/N ratios of 2 - 10 obtaining an optimum value at 6.11 using a substrate with high N content as soybean bran. Gombert et al. (1999) studied the influence of C/N ratio on lipase production by *Penicillium restrictum,* and achieved 30.3 U g^{-1} using babassu oil cake with C/N ratio of 14.1. The present work achieved highest lipase activities without the need for reduction of C/N ratio to levels of 2 - 15. Thus, this gives the opportunity to use residues with low N content with low amount of N sources supplementation.

The lipids content can be one of the most important factors influencing the production of lipase in SSF. OP contains residual olive oil that acts as inducer on lipase production. To evaluate

the effect of lipids in lipase production, several mixtures of OP:WB and WB:olive oil were studied, which led to a wide range of lipids concentrations (3.66 - 16.7%). The maximum lipase production was achieved between 10.2 - 13.7% of lipids. As can be seen, the addition of 10% of olive oil to WB led to a lipase production of 152 ± 3 U g^{-1}, that is similar to the one obtained with the OP:WB in a ratio of 1:1. Other researchers also found that olive oil induces the production of lipase in SSF. For example, Palma et al. (2000) found a positive effect of the addition of 1% olive oil to babassu cake for lipase production by *Penicillium restrictum*, achieving 17.2 U g^{-1}, a 1.7-fold increase compared to no-supplemented substrate. Also, Falony et al. (2006) found a positive effect of adding 1.5% olive oil to WB, obtaining 9.1 U g^{-1} of lipase.

Table 4.2 – Results of experimental values of lipase activity (LA) from SSF performed with different ratios of olive pomace (OP) and wheat bran (WB) and from SSF performed with WB supplemented with different concentrations of olive oil; and respective variation on C/N ratio, lipids, total phenols and reducing sugars content. LA values are the mean of triplicate analysis ± standard deviation (SD).

OP:WB ratio	C/N ratio	Olive oil addition (%)	Lipids (%)	Phenols (%)	Reducing sugars (%)	Experimental LA ± SD (U g^{-1})
1:0	56.5		16.7	0.84	9.6	3.6 ± 1.1
3:2	28.5		11.5	0.66	6.4	122 ± 2
1:1	25.2		10.2	0.61	5.6	144 ± 5
2:3	22.5		8.9	0.56	4.7	142 ± 2
1:4	18.5		6.3	0.47	3.1	109 ± 3
0:1	15.5	0	3.7	0.38	1.5	84 ± 6
0:1	15.8	1	4.7	0.38	1.5	93 ± 7
0:1	16.1	2.5	6.2	0.38	1.5	104 ± 4
0:1	16.7	5	8.7	0.38	1.5	112 ± 5
0:1	17.8	10	13.7	0.38	1.5	152 ± 3

However, high concentrations of olive oil can limit oxygen transfer which could modify the microbial metabolism leading to less lipase production (Amin and Bhatti, 2014). In this sense, Damaso et al. (2008) observed a reduction of lipase production by *A. niger* with increasing olive oil addition to WB, and at 12% of olive oil lipase activity was no longer detected. The high lipids content in OP could be one of the reasons for the low lipase activity in substrates with elevated OP:WB ratio (1:0 and 4:1). Thus, the mixture of OP with other agro-industrial residues as WB can adjust the content of lipids to an optimum value for lipase production. These results showed the

importance of the use of agro-industrial residues containing lipids as well as the mixture of them in order to improve lipase production.

The multiple linear regression analysis was used to describe the effect of these parameters on lipase production. In addition, other independent variables were evaluated as total phenols and reducing sugar contents. These parameters can also affect the growth of fungus and the production of enzymes (Leite et al., 2016). On the basis of statistical parameters showed in Table 4.3, the model Eq. (4.1) showed a good fit, since the value of the coefficient of determination (R^2) was 0.9758. This value indicates that the model explains 97.58% of the variability in lipase production.

$$LA = 158.24 - 8.54\ CNR + 8.40\ LP + 19.43\ RS \quad (4.1)$$

Where LA is lipase activity (U g^{-1}), CNR is the C/N ratio, LP is lipids concentration (%, w/w), and RS is the reducing sugars concentration (%, w/w). All independent variables were statistically significant at 99% of confidence level except the content in total phenols that was not significant, thus it was no taken into account in the model.

Table 4.3 – Analysis of variance (ANOVA) for the regression model representing lipase activity.

Source	SS	df	MS	*F*-ratio	*p*-value
Model	15886.5	3	5295.52	80.55	0
Residual	394.452	6	65.742		
Total (Corr.)	16281	9			
R^2 = 97.58%					
R^2 (adjusted for df) = 96.37%					
Standard Error of Est. = 8.11					
Mean absolute error = 4.9					

R^2 – coefficient of determination, SS – sum of squares, df – degrees of freedom, MS – mean squares

Figure 4.3 showed the experimental values versus model outputs for dependent variables, thus obtaining a heuristic evaluation of the performance. On basis of these results, lipase production by *A. ibericus* can be predicted knowing the composition of lipids, C/N ratio and sugars

content. This is interesting, especially in the use of OP as solid substrate, since its composition vary in function of olive oil extraction conditions used (Alburquerque et al., 2004).

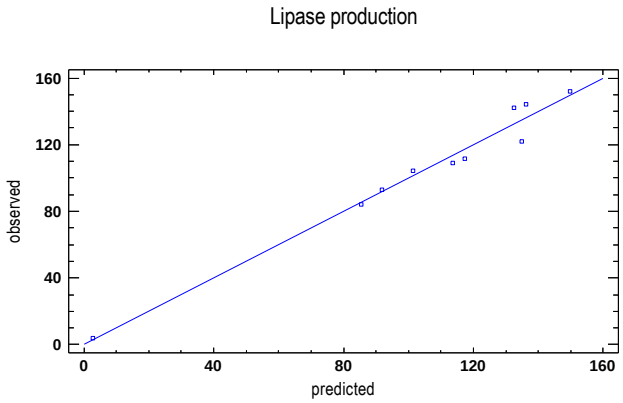

Figure 4.3 – Graphical representation of the observed and predicted values of lipase activity obtained, using the test data set.

4.3.1.3 Time course of lipase production

Figure 4.4 presents results of lipase activity and its productivity over fermentation time. An increase of lipase production over time was observed, reaching 166 ± 5 U g^{-1} and 209 ± 10 U g^{-1} after 10 and 20 days of fermentation, respectively.

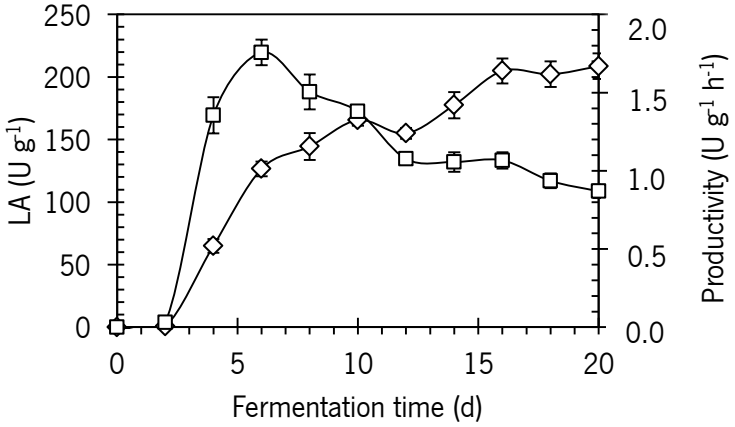

Figure 4.4 – Time course profiles of (—◇—) lipase activity (LA) and (—□—) productivity in SSF conducted at optimum conditions. Depicted values are the mean of triplicate analysis ± standard deviation.

However, maximum productivity was obtained on the 6th day (1.8 ± 0.1 U g^{-1} h^{-1}) with a lipase production of 127 ± 6 U g^{-1}. Using the same strain on OP at different SSF conditions, Salgado et al. (2014b) found maximum lipase production on the 5th day, after which a stabilisation over 12 days occurred. On the contrary, some authors observed maximum lipase production on the 3rd day (Gutarra et al., 2009), the 4th (Edwinoliver et al., 2010) and the 5th day (Mahadik et al., 2002) of fermentation, with *P. simplicissimum* on babassu cake with sugarcane molasses, *A. niger* on wheat rawa, coconut oil cake and WB, and *A. niger* on WB, respectively. After the maximum, they also observed a decline with time course.

4.3.2 SSF in a packed-bed bioreactor

Different aeration rates were evaluated in a packed-bed bioreactor for SSF. Results are presented in Figure 4.5. It was observed visually the dehydration of the substrate at the beginning of the column over fermentation time, even using saturated air. Highest values of lipase production were obtained at aeration rates of 0.05 L min^{-1} and 0.1 L min^{-1}, using 25 g of substrate (2 mL min^{-1} g^{-1} and 4 mL min^{-1} g^{-1}, respectively), without statistically significant differences among the results at both conditions. A SSF without aeration was performed, where no fungal growth was observed. The column was completely filled and maintained without aeration, thus limiting fungal growth due to the lack of oxygen.

The aeration rate favours the transport of oxygen to the solid substrate. However, at very high aeration rates, lipase activity may decrease due to the fungal metabolism changes (Díaz et al., 2013). Reduction of lipase observed at the 2 L min^{-1} could be attributed by excessive forced aeration used and by dehydration along the column derived from that. Results of lipase produced in a packed-bed bioreactor, using 0.05 L min^{-1}, were statistically similar to that ($p > 0.05$) obtained in flasks (144 ± 5 U g^{-1} – Table 4.2), after 7 days of SSF.

Pérez-Rodríguez et al. (2014) found an optimum aeration rate of 0.1 L min^{-1} in a packed-bed bioreactor using 20 g corncob (5 mL min^{-1} g^{-1}) for xylanase production by SSF with *A. niger*. Using the same bioreactor, Salgado et al. (2015) found an optimum aeration of 0.2 L min^{-1} using 20 g of mixtures of winery and olive mill wastes (10 mL min^{-1} g^{-1}) which improved cellulase and xylanase production by *A. uvarum*. In the production of citric acid using *A. niger*, Lu et al. (1997) reported

the need of an aeration rate of 1.5 L min^{-1} in a packed-bed using 180 g of kumara (starch root crop) (8.3 mL min^{-1} g^{-1}) to maximise acid production.

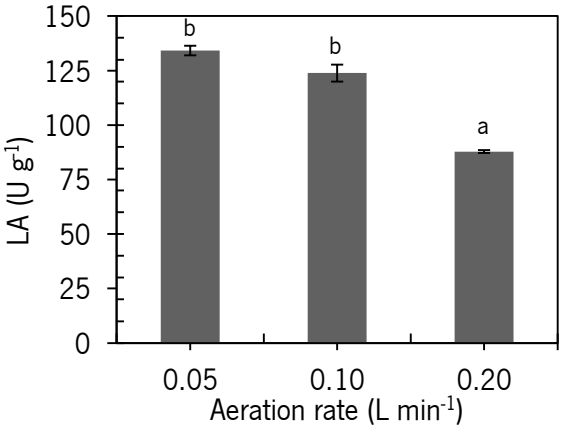

Figure 4.5 – Results of lipase activity (LA) of SSF in a packed-bed bioreactor, at different aeration rates. Values are the mean of three independent fermentation experiments ± standard deviation. Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

4.3.3 Optimisation of lipase extraction

The efficacy of the enzyme extraction is very important. In order to extract maximum lipase from the fermented solid substrates, the efficacy of lipase extraction procedure was tested assessing the effect of the type of solvent and then the best volume of solvent. The highest lipase activities were recovered when the fermented substrate was extracted with 7.5 mL of 10 g L^{-1} Triton X-100 per g of dry solid substrate (Table 4.4). Thus, Triton X-100 aqueous solution appeared to be the best solvent solution and the role of NaCl on that extracting solution is negligible. Triton X-100 is a non-ionic surfactant and it might solubilise the enzyme from the solid substrate to the emulsion, resulting in highest recoveries of lipase. In the work from chapter 3 and also in this work, 5 g L^{-1} Triton X-100 with 10 g L^{-1} NaCl was used for extraction, based on literature (Edwinoliver et al., 2010; Mahadik et al., 2002). However, this study showed that NaCl is not needed on lipase extracting solution and a minimum of 10 g L^{-1} Triton X-100 was needed to 1.2-fold increase of lipase extracted. Similarly to these results, some authors also found 10 g L^{-1} Triton X-100 as the best solvent for lipase extraction in SSF (Balaji and Ebenezer, 2008; Rodriguez et al., 2006).

Also, the increase of the volume of extraction solvent to 7.5 mL per gram of dry solid substrate led to the improvement of lipase extraction, leading to a 1.5-fold increase in lipase activity, in comparison to initial extraction conditions. Pal and Khanum (2010) found an optimum solvent volume of 10 mL g^{-1} for the extraction of *A. niger* xylanase, while Díaz et al. (2007) found that the use of 5 mL g^{-1} of solvent were most appropriate for extracting xylanase.

Table 4.4 – Conditions of extraction and respective lipase activity (LA). Values are the mean of triplicate analysis ± standard deviation (SD). Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

Effect of extraction solvent type – 5 mL g^{-1}, 2 h, 170 rpm, 20 °C	
Solvent (5 mL g^{-1})	LA ± SD (U g^{-1})
Distilled water	17 ± 1^{b}
Phosphate buffer	23 ± 1^{bc}
1% NaCl	5 ± 2a
1% Tween 80	30 ± 3^{c}
0.5% triton + 1% NaCl	144 ± 5^{d}
1% Triton + 1% NaCl	153 ± 6^{d}
0.5% Triton	147 ± 9^{d}
1% Triton	175 ± 5^{e}
2% Triton	161 ± 4^{e}

Effect of extraction volume – 1% Triton, 2 h, 170 rpm, 20 °C	
Volume (mL g^{-1})	LA ± SD (U g^{-1})
2.5	77 ± 3^{a}
5	156 ± 3^{b}
7.5	230 ± 6^{c}

Consecutive extractions of the fermented substrate were also performed in order to determine the lipase recovery in the extraction, at optimum extraction conditions. Table 4.5 presents lipase activity and respective percentage of lipase recovery of those experiments. The first extraction yielded a lipase activity of 223 ± 5 U g^{-1}, which corresponds to a lipase recovery of 76%. With a second extraction, it was possible to extract almost all the remaining lipase contained in the fermented substrate. Results agreed with Rodriguez et al. (2006) who obtained a lipase recovery of 70% in the 1st extraction, using 10 g L^{-1} Triton X-100.

Table 4.5 – Results of lipase activity (LA) and respective lipase recovery (LR) from consecutive extractions of the fermented substrate. Values are the mean of triplicate analysis ± standard deviation (SD). Means within the same line with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

	Number of consecutive extractions				
	1st	2nd	3rd	4th	5th
LA ± SD (U g^{-1})	223 ± 5^{c}	65 ± 2^{b}	3.5 ± 0.2^{a}	0.9 ± 0^{a}	0.5 ± 0^{a}
LR ± SD (%)	76.1 ± 0.2^{d}	22.2 ± 0.3^{c}	1.2 ± 0.1^{b}	0.3 ± 0^{a}	0.2 ± 0^{a}

4.4 Conclusions

The present study allowed to improve the lipase production by SSF of mixtures of OP+WB by optimisation of substrate composition and lipase extraction. The optimised substrate had C/N ratios close to 25 and lipids content of 10 - 13%. These conditions were achieved by a mixture of OP and WB (1:1), which was used directly without treatment and MC adjustment. In addition, the nutritional supplementation was minimised by using ammonium sulphate at a concentration of 1.33% (w/w) to the mixture.

The optimised medium was successfully tested in a lab-scale packed-bed bioreactor, thus it is a promising system for lipase production at industrial scale. The study also demonstrated that the extraction of lipase is a key factor in SSF which can improve the final lipase concentration in the extract.

5 Scale-up of lipase production by solid-state fermentation of olive pomace in tray-type and pilot-scale pressure bioreactors

The scale-up of lipase production by solid-state fermentation (SSF) of olive pomace (OP) was evaluated in a pressurised bioreactor and compared with traditional tray-type bioreactor. Important aspects for scale-up of SSF were studied, such as the need of sterilisation and moisture content (MC) control.

In larger scale, there was no significant difference in lipase production between sterilised and no-sterilised substrates, but MC control had significant impact. The production of lipase in a pressurised bioreactor, under air pressures of 200 kPa and 400 kPa, was twice higher than in tray-type bioreactor using the same amount of substrate (500 g) and the same bed height.

A. ibericus lipase presented maximum activity at 50 °C and pH 7.0. Also, at room temperature, lipase activity was retained for 3 days, with a relative activity higher than 80% of the initial value. Lyophilisation of enzymatic extracts may be used as a way to preserve lipase, since a relative activity of 80 ± 7% was maintained after solubilisation, and an activity of 1000 U g^{-1} of lyophilised enzymatic extract was found.

The protein content of substrate increased from 10 to 18% (w/w) after SSF and the fermented material presented an interesting antioxidant activity (10 mmol Trolox kg^{-1}). This improvement of nutritional quality of the substrate by SSF indicates its potential applicability in animal feed.

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Oliveira, F., Venâncio, A., Belo, I., Pérez-Rodríguez, N., & Domínguez, J. M. (2015). Scale-up of *Aspergillus ibericus* lipase production by solid-state fermentation. In C. Vilarinho, F. Castro, & M. Russo (Eds.), *Wastes: Solutions, Treatments and Opportunities* (Taylor & Francis Group), pp. 203–208. London. ISBN: 978-1-138-02882-1. (Selected papers from the 3rd edition of the International Conferences on Wastes: Solutions, Treatments and Opportunities, Viana do Castelo, Portugal, 14-16 September).

5.1 Introduction

Due to low water activity in solid-state fermentation (SSF), lower demand on sterility is needed (Singhania et al., 2009). The sterilisation of substrate is a challenge in the scale-up of SSF to industrial development. The difficulty of filling the bioreactors with solid substrate in sterile conditions makes it essential to use sterile-in-place bioreactors, which increases the costs of process. On the other hand, SSF offers the opportunity of using low cost agro-industrial residues for metabolites production (Pandey, 2003). In SSF processes at industrial level, the growth of microorganism and the production of metabolites may be limited by heat transfer and by mass transfer of oxygen and nutrients, depending on the location in the substrate bed, the stage of the fermentation, and also by the design and operation of the bioreactor (Mitchell et al., 2000). Different bioreactor types have been used in SSF, including tray-type, packed-bed and horizontal rotary-drum, presenting each one their own advantages and disadvantages (Singhania et al., 2009).

Pressurised bioreactors may overcome some of the previous problems, and it was hardly studied in SSF. Pressurised air can be successfully applied to cultivations, as a way of improving the oxygen transfer rate to aerobic cultures (Belo et al., 2003). However, above certain limits the increased air pressure and the consequent increase in oxygen partial pressure may have detrimental effects on cells activity and metabolite production (Belo et al., 2005). Lopes et al. (2009, 2008) reported significant improvements on lipase productivity by a nonconventional yeast, *Yarrowia lipolytica*, by submerged fermentation (SmF), using air pressure up to 600 kPa. Air pressure increased by pulses has been used to enhance mass and heat transfer in SSF, stimulating the growth and metabolism of microorganisms (Xu et al., 2002), as well as the production of different enzymes (Aijun et al., 2005; Chen et al., 2014; Zeng and Chen, 2009; Zhao et al., 2001).

The bioconversion of the agro-industrial residues for lipase production occupies a prominent position, mainly due to the possibility of obtaining the biocatalyst at lower costs using environmentally friendly techniques (Fleuri et al., 2014). Moreover, the use of SSF as way to obtain the enzyme is an interesting strategy, since higher yields are found and the product is more stable than the obtained from SmF (Longo et al., 2008). Lipases have many biotechnological applications, as in the food industry, detergent, fine chemicals, and other industries (Fleuri et al., 2014). Lipases

used in different biochemical reactions are selected based on their substrate specificity, as well as their optimum temperature and pH, and stability (Gutarra et al., 2009).

For the other hand, SSF of agro-industrial residues is a promising alternative to improve the nutritional value to these by-products, allowing its use for animal feed (Canedo et al., 2016). In addition to producing enzymes such as lipases, the fungal growth may increase protein content of the residues; it may increase the biodegradability facilitating the digestibility by ruminant animals; and it may reduce the content of polyphenols that can affect their palatability (Salgado et al., 2015). In this way, all fractions after SSF can be exploited by agro-food industries.

The aim of this work was to study the scale-up of SSF for lipase production using a novel pressurised bioreactor and a traditional tray-type bioreactor. Different aspects were evaluated as the need of sterilisation, moisture content (MC) control, the substrate bed height and the air pressure and aeration rates. In addition, lipase produced was characterised with regard to optimum temperature and pH conditions, and it was evaluated the performance of the enzyme after been lyophilised, to assess the applicability of this method for lipase preservation. Additionally, the composition of fermented substrate was evaluated for its applicability as animal feed.

5.2 Materials and methods

5.2.1 Microorganism

Aspergillus ibericus MUM 03.49 was used, as described in chapter 4.2.1.

5.2.2 Substrates

Olive pomace (OP) from campaign 2013/2014 and wheat bran (WB) were used, similarly to the chapter 4.2.2. Substrates were used without any pre-treatment. Characterisation of OP and WB is presented in Table 5.3.

5.2.3 SSF of OP with WB

5.2.3.1 SSF in flasks

SSFs experiments were performed using OP mixed with WB, in a ratio of 1:1 (w/w, dry basis), 0.0133 g of $(NH_4)_2SO_4$ per gram of dry solid substrate, 33.33 μL g^{-1} of 10^7 spores mL^{-1} inoculum suspension. The mixture of OP with WB resulted in optimum MC, of around 60% (w/w), without the need for its adjustment. Fermentations were carried out at 30 °C during 7 days without agitation (SSF optimisation from chapter 3 and chapter 4). SSFs were performed using sterilised (121 °C, 200 kPa for 15 min) and no-sterilised substrate.

SSF in cotton-plugged 500 mL Erlenmeyer´s flasks were performed using 30 g of dry solid substrate. SSFs were performed in triplicate, for experiments with and without sterilisation. A SSF experiment using no-sterilised and non-inoculated substrate was used as control.

5.2.4 SSF in tray-type bioreactor

The tray-type bioreactor used consisted in a vertical incubator (112 x 48 x 45 cm) containing four (no perforated) stainless steel trays (38 x 26 x 5 cm). SSFs were carried out in the 4 trays at the same conditions (Figure 5.1). SSFs were performed using sterilised (121 °C, 200 kPa for 15 min) and no-sterilised substrate, in quadruplicate. Each tray was filled with 300 g of dry solid substrate, cooled and inoculated, resulting in a substrate bed height of 2.5 cm. The incubator was opened once a day for monitoring and to allow aeration. In SSF with MC control, MC was adjusted by adding sterile distilled water in the same amount of the weight loss of each tray, once a day.

Another SSF was performed using 500 g of dry solid substrate in (no perforated) smaller trays (20 x 25 x 10 cm), and covered with a polymeric (polyethylene) film, to avoid MC reduction. The substrate was not sterilised and it was inoculated. A substrate bed height of 8 cm was obtained. Temperature inside substrate was monitored in all of the experiments performed in tray-type bioreactor.

Figure 5.1 – Tray-type bioreactor containing fermented substrate.

5.2.5 SSF in pilot-scale pressure bioreactor

SSFs were carried out in 19 L (42 cm height and 24 cm diameter) stainless steel stirred tank bioreactor (4555, Parr Instrument Company, USA) (Figure 5.2). SSFs were performed using 500 g of dry solid substrate no-sterilised. The substrate bed height formed was around 8 cm. Absolute air pressures of 200 kPa, 400 kPa and 700 kPa were selected by the inlet air pressure setting and by controlling the regulatory inlet and outlet air valves. Continuous air sparging was performed at the bottom of the bioreactor at aeration rates of 1 L min^{-1} and 2 L min^{-1}, measured at the outlet gas conditions. SSF experiments were performed in duplicate.

Figure 5.2 – Pilot-scale pressure bioreactor (4555, Parr Instrument Company, USA).

5.2.6 Enzymes extraction and determination

Before extraction, samples (1.5 g) of the fermented substrate were taken for MC determination. Lipase extraction was performed as described in chapter 3.2.6, using 10 g L^{-1} Triton X-100. Lipase activity was determined by a spectrophotometric method, as described in chapter 4.2.5.

The protein content of the extract was determined by Bradford´s method, using BSA as the standard (Bradford, 1976). Protein concentration was expressed as mg of protein per gram of dry solid substrate (mg g^{-1}). Specific activity was obtained by the ratio between lipase activity and protein concentration. It was expressed as units of lipase activity per milligram of total protein (U mg^{-1}). All analyses were performed in triplicate.

Other enzymes, such as xylanase, cellulase and β-glucosidase were determined in the enzymatic extract after 7 days of SSF using sterilised substrate. Enzymes determination were performed as described by Salgado et al. (2015). Cellulase (endo-1,4-β-glucanase) activity was analysed using an enzymatic kit Azo-CM-Cellulase S-ACMC 04/07 (Megazyme International, Ireland). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of glucose reducing sugar equivalents from CM-cellulose in 1 min at pH 4.5. Xylanase (endo-1,4-β-xylanase) activity was analysed using an enzymatic kit Azo wheat arabinoxylan AWX 10/2002 (Megazyme International, Ireland). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of xylose reducing sugar equivalents from wheat arabinoxylan in 1 min at pH 4.5. β-Glucosidase activity was estimated using pNPG as substrate. The assay mixture containing 25 µL of substrate [5 mM, 4-nitrophenyl β-D-glucopyranoside (pNPG)], 25 µL of diluted enzyme, and 50 µL of acetate buffer (50 mM, pH 5.0) was incubated at 50 °C for 30 min, and the *p*-nitrophenol (pNP) liberated was measured at 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of pNP per minute under standard assay conditions. Enzymes activity obtained were expressed as units per gram of dry solid substrate (U g^{-1}).

Lignin peroxidase, manganese peroxidase and laccase were determined in the enzymatic extract after 7 days of SSF using sterilised substrate. Enzymes determination were performed as described by Salgado et al. (2014a). One unit of lignin peroxidase activity represented the oxidation of 1 µmol of veratryl alcohol per minute at 30 °C. Manganese peroxidase activity was determined

by oxidation of Mn^{2+} to Mn^{3+}, measuring the increase of absorbance at 270 nm. One unit of manganese peroxidase activity was defined as the amount of enzyme that produced 1 µmol of Mn^{3+} per minute at 25 °C. Laccase activity was determined by spectrophotometric method, based on monitoring the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) by measuring the increase of absorbance at 420 nm. One unit of laccase activity corresponded to oxidation of 1 µmol of ABTS per minute at 25 °C.

5.2.7 Lipase characterisation

5.2.7.1 Lyophilisation of enzymatic extracts and resuspension

Frozen enzymatic extracts obtained from SSF in flasks using sterilised substrate were used. Volumes (20 mL) of frozen enzymatic extracts were lyophilised and ressuspended in potassium phosphate 50 mM at pH 7.0 or in distilled water in the same initial volume. Lyophilisation of samples was performed in duplicate.

5.2.7.2 Effect of temperature and pH on lipase activity

Lipase samples (liquid enzymatic extract, lyophilised extract containing lipase and ressuspended in buffer, as well as ressuspended in distilled water) were used to characterise the hydrolytic activity of *A. ibericus* lipase from SSF of OP with WB. Lipase activity dependence of temperature was determined at different incubation temperatures and at pH 7, under the standard assay conditions. The effect of pH on lipase activity was assessed using potassium phosphate 50 mM at different pH values and at 37 °C, under the standard assay conditions. Molar extinction coefficients of pNP were determined at different temperatures (at pH 7) and at different pH (at 37 °C) and are presented in Table 5.1.

Table 5.1 – Results of molar extinction coefficients (ε) of *p*-nitrophenol (pNP) as affected by temperature and pH.

Temperature (°C)	20	30	37	40	50	60
ε (mM^{-1}) (at pH 7)	8.2396	8.9958	9.4169	9.5948	10.0520	10.7800
pH	5.7	6.0	6.5	7.0	7.5	8.0
ε (mM^{-1}) (at 37 °C)	1.0606	1.8116	4.3844	9.4169	13.2057	16.1193

5.2.7.3 Lipase stability at room temperature

Lipase samples (liquid enzymatic extract, lipase lyophilised and ressuspended in buffer, lipase lyophilised and ressuspended in distilled water) were exposed at room temperature (20 °C) during 3 days, in order to characterise the long term stability. Lipase activity was assayed every day, under the standard assay conditions.

5.2.8 Characterisation of substrates before and after SSF

The composition of substrates before and after SSF was determined to evaluate the increase of nutritional quality and potential applicability as animal feed. Initial characterisation of OP was performed by Leite et al. (2016) and is presented in Table 5.3. Characteristics of WB and of the fermented substrate (SSF OP+WB) such as MC, total solids, lignin, hemicellulose, cellulose, lipids, protein concentration, reducing sugars, phenols, nitrogen and carbon, were determined according to Leite et al. (2016), and is described in chapter 3.2.2. Ash, crude protein and metals also were determined by Leite et al. (2016).

Ash was determined by weight loss of dry solids through ignition in a muffle at 550 °C for 2 h. Crude protein was determined by multiplying total nitrogen content by a factor of 6.25 (Salgado et al., 2015). Metals, such as Ca, Mg, Zn, Cu, Fe, Mn, Cr, Ni, Pb, Na, and K were analysed in ashes using flame atomic absorption spectrometry (FAAS) and flame atomic emission spectrometry (FAES) using a Varian SpectrAA-220. Previously, 0.15 g of ashes was digested with 5 mL of 65% HNO_3, 1 mL of 30% H_2O_2, and 0.5 mL of 40% HF in a Microwave Labstation MLS 1200 MEGA, MILESTONE (Italy). The analyses were carried out using an air/acetylene flame. FAAS was used to

analyse Ca, Mg, Zin, Cu, Fe, Mn, Cr, Ni, and Pb, and FAES was used to determine Na and K (Salgado et al., 2015).

The extracts for antioxidant determination in unfermented and fermented substrates were obtained by methanol extraction with ratio liquid:solid of 20:1 (v/w). The extraction was carried out in pyrex bottles in an incubator shaker at 50 °C, 100 rpm and 1 h, and the extracts were filtered with filter paper Whatman. Antioxidant activity was determined with 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method in microplate described by Dulf et al. (2015). Known amounts of Trolox were used for calibration. The free radical scavenging activity of the extracts was expressed as millimoles of Trolox equivalents per kilogram dry solid substrate (mmol kg^{-1}).

To observe modifications in concentration of compounds, in relation to the initial value, it was determined an average of characteristics between OP and WB, since the ratio (dry basis) used in SSF was 1:1 (w/w). A characteristic increase (or reduction) in relation to the initial value, was determined by the Eq. (5.1), as follows:

$$XIR = (X_f - X_i) / X_i \times 100 \quad (5.1)$$

Where XIR is a characteristic increase (or reduction) expressed in percentage (%), X_i is the initial value of a characteristic (before SSF), and X_f is the final value of a characteristic (after 7 days of SSF).

5.2.9 Statistical treatment

Data obtained were statistically analysed using SPSS, as described in chapter 3.2.7.

5.3 Results and discussion

5.3.1 Effect of sterilisation on lipase production by SSF in small scale

The evaluation of sterilisation effect on lipase production was firstly carried out at small scale in Erlenmeyer´s flasks using sterilised and no-sterilised inoculated substrates. A control experiment was performed with no-sterilised and non-inoculated substrate. The growth of *A. ibericus* was observed visually in both inoculated substrates. In the control assay, it was observed the growth of different autochthonous microorganisms producing different green and brown colonies, unlike the black spores observed in the flasks inoculated with *A. ibericus*. In this sense, the sterilisation of the substrate removed the wild microbial population, contributing to a better colonisation with the *Aspergillus* strain used as inoculum.

As can be observed in Figure 5.3, lipase production was higher when the substrate was sterilised. In flasks using no-sterilised substrate, 34% and 40% significant reduction of lipase production and specific activity was observed, respectively. For another hand, in the control experiment (no-sterilised and non-inoculated) a considerable lower lipase production was achieved. The sterilisation and the inoculation with *A. ibericus* spores on OP and WB mixture led to 2.7-fold increase in lipase production, compared to the fermentation control.

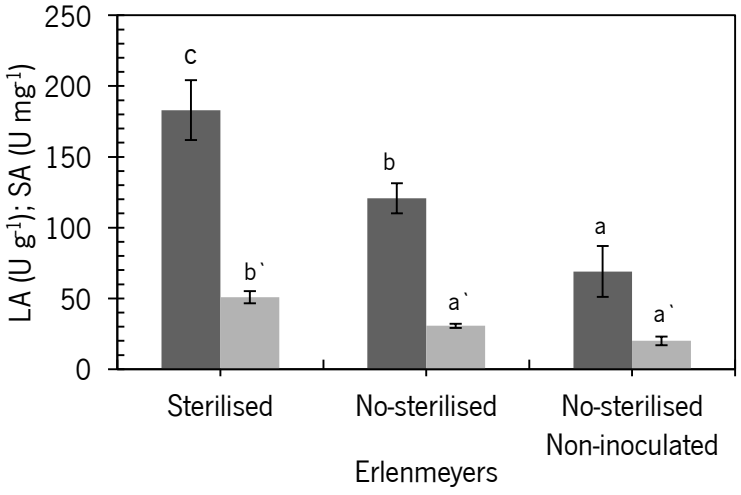

Figure 5.3 – Results of (■) lipase activity (LA) and (■) specific activity (SA) of SSF in 500 mL Erlenmeyer´s flasks, using sterilised and no-sterilised substrate, and using no-sterilised and non-inoculated substrate. Values are the mean of three independent fermentation experiments ± standard deviation. Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

The positive effect of substrate sterilisation in small scale fermentations may also be due to the modification of solid structure by heat, which lead to the formation of micropores in the substrate, representing an increment of the specific surface area (Pérez-Rodríguez et al., 2014), improving the water solubility and water binding properties (Dundar and Gocmen, 2013). Consequently, it may increase the accessibility of the fungus and its enzymes to the micro and macronutrients of the substrate (OP+WB). For these reasons, sterilisation may be considered as a pre-treatment that may increase the enzymes production. In this sense, Pensupa et al. (2013) compared autoclave sterilisation of wheat straw with common chemical treatments, as dilute acid and acid soaking, to produce cellulase by SSF. They observed that the autoclave sterilisation increased the enzymes production, but there was no significant difference between acid and heat treatments.

5.3.2 SSF scale-up to tray-type bioreactor

SSF was scaled-up from flasks (30 g) to the tray-type bioreactor using 300 g of dry solid substrate, with and without MC control, using sterilised substrate. In SSF without MC control, the rate of the MC loss of the trays was higher in the first days of fermentation and decreased with time. After 7 days of fermentation in the tray-type bioreactor (Table 5.2), lower lipase activity was obtained, since MC decreased 55% (from 60% to 27% (w/w)). Whereas, in SSF with MC periodic control, higher lipase production was obtained leading to 50% increase of lipase activity and 63% increase of specific activity. The MC periodic control to optimum levels, of around 60% (as observed in chapter 3), minimises the negative effects of low MC, such as the reduction in solubility of nutrients of the substrate (Lonsane et al., 1985).

The 10-fold scale increase of SSF from flasks to tray-type bioreactor with MC periodic control in sterile conditions, led to 40% significant decrease in lipase production and 29% decrease of specific activity. This decrease could be caused by the slight dehydration of the substrate (an average of 10% MC loss) along each day of SSF, but also by the slightly higher substrate bed height (2.5 cm) than the used in flasks (2 cm), leading to the substrate temperature rise during the fungus growth. In fact, temperature was measured in the tray-type bioreactor over fermentation time and an increase of temperature till 34.3 °C was observed after 1 day of fermentation. The

increase of temperature above optimal level for lipase production by *A. ibericus* may have a negative impact on final activity, as observed in chapter 3.

Table 5.2 – Results of lipase activity (LA), specific activity (SA) and final moisture content (MC), from SSF in a tray-type bioreactor, with and without MC control, and using no-sterilised and sterilised substrate. Values are the mean of four trays ± standard deviation (SD). Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

SSF conditions	LA ± SD (U g^{-1})	SA ± SD (U mg^{-1})	MC ± SD (%, w/w)
Without MC control, sterilised	74 ± 10^{a}	22 ± 2^{a}	27 ± 3^{a}
With MC control:			
Sterilised substrate	111 ± 14^{b}	36 ± 6^{b}	66 ± 3^{b}
No-sterilised substrate	131 ± 16^{b}	25 ± 4^{a}	67 ± 3^{b}

In general, the increase of substrate bed height had significant negative impact in lipase production in the tray-type bioreactor. Edwinoliver et al. (2010) scaled-up the lipase production from flasks with 10 g of substrate to tray-type bioreactor with 100 g (0.8 cm substrate bed height) and 1 kg (1 cm substrate bed height), and reported a reduction of 4% and 17%, respectively, of the lipase produced by *A. niger* on WB, coconut oil cake and wheat raw. The decrease of lipase activity was attributed to the temperature increase during the culture growth. Vaseghi et al. (2013) studied the effect of using substrate bed height between 0.5 cm and 3 cm and reported an optimum substrate bed height of 0.5 cm for SSF on sugarcane bagasse for *Rhizopus oryzae* lipase production on tray-type bioreactor, yielding around 200 U g^{-1}. Rajagopalan and Modak (1994) suggested that the substrate bed height should be lower by increasing the area of the tray, to prevent temperature gradients in order to maximise lipase production.

In the SSF experiments with MC control with 300 g of substrate, there was no significant impact of the substrate sterilisation (Table 5.2). El-Bakry et al. (2016) also achieved high protease production in non-sterile conditions in SSF with 110 g and 2300 g of solid substrate, but in this case, the use of thermophilic microorganisms allowed operating at high temperatures (55 °C) that may have limited the growth of other microorganisms.

5.3.3 SSF scale-up to pressurised bioreactor

SSF process in a pressurised bioreactor was performed with 500 g of no-sterilised substrate. Figure 5.4 presents results of SSF in the pressurised bioreactor, at aeration rate of 1 L min^{-1} and 2 L min^{-1}, and at different absolute air pressures. Maximum lipase activity was found at lower air pressures of 200 kPa and 400 kPa, without statistical difference between those results. However, the maximum specific activity was obtained at 200 kPa. Results of lipase and specific activities were independent of aeration rate used, since no significant differences were found among results of SSF at aeration rate of 1 L min^{-1} or 2 L min^{-1}.

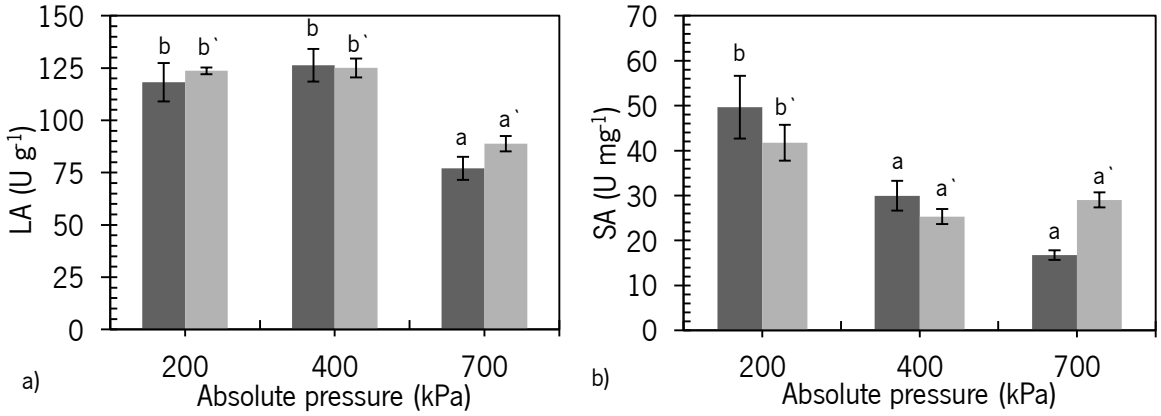

Figure 5.4 – Results of a) lipase activity (LA) and b) specific activity (SA) of SSF of no-sterilised substrate in a pressurised bioreactor at different absolute pressures and at (■) 1 L min^{-1} and (■) 2 L min^{-1} of aeration rate. Values are the mean of two independent fermentation experiments ± standard deviation. Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

The results of lipase activity obtained in the pressurised bioreactor using no-sterilised substrate, under 200 or 400 kPa and 1 L min^{-1} or 2 L min^{-1} were statistically equal to the lipase obtained in flasks with no-sterilised substrate. However, about 60% significant increase was observed for specific activity, indicating that pressure increase may be an important operating factor for optimisation of enzymes production, since it may affect differently several enzymes expression.

In order to compare the results obtained in the pressurised bioreactor with tray-type, SSF was performed in trays, using the same amount of substrate (500 g) and bed height of 8 cm. The trays were covered with a perforated polymeric film in order to simulate the close environment as

in the pressurised bioreactor. The coverage of the tray prevented MC loss. The lipase production obtained (61 ± 13 U g^{-1}) was significantly lower (around 50%) than all the values obtained under the other conditions tested. This result may be attributed to the temperature rise inside the substrate bed that increased dramatically after 1 day of fermentation to around 36 °C. Moreover mycelium growth was observed in all the substrate in the trays but sporulation occurred only at the substrate surface indicating oxygen limitations through substrate bed.

Contrarily, in the pressurised bioreactor fungus growth and sporulation was observed in all substrate, at all the conditions tested. The continuous aeration of pressurised air in this bioreactor may allow an efficient gas exchange over fermentation time and the dissipation of excessive heat derived from fungus growth, even using a high substrate bed height. This proved that the use of pressurised bioreactor may be effective to maintain lipase production with high bed heights.

Lopes et al. (2008) showed that for aerobic cultures, the total air pressure is a key controlling factor, as it alters the dissolved gas partial pressure, in particular the dissolved oxygen, which may be a limiting factor for cell growth and metabolic activity. And, the effect of pressure on the microorganism metabolism depends of the culture conditions, pressurisation mode and the microorganism itself.

SSF under increased air pressure has been fairly explored and the known works used air pressure pulsation instead of continuously air pressure exposure as it was used in the herein work. Aijun et al. (2005) applied air pressure pulsation SSF, and found that the protease of *Bacillus pumilus* increased in relation to static SSF, when pressure amplitudes used were 50 kPa and 100 kPa. This process supplied sufficient oxygen and enlarged aerobic surface for the microorganism growth (Aijun et al., 2005). Similarly, Zeng and Chen (2009) applied cycles of 20 min without pressure increase and 10 s pulses of air at 200 kPa in SSF, and observed that feruloyl esterase production by *A. niger* was improved and also the fermentation time to achieve the highest enzyme production was shortened, comparing to static SSF.

This work demonstrated that SSF under pressurised air could improve specific activity. However, using higher pressures (700 kPa) the lipase activity decreased, probably due to unbalanced oxidative stress. To our knowledge, this is the first report on the study of lipase production by *A. ibericus* under continuous pressurised aeration conditions through a SSF process.

5.3.4 Lipase characterisation

5.3.4.1 Temperature and pH effect

By Figure 5.5 it can be seen that maximum lipase activity was found at 50 °C and at pH 7. Lipase achieved higher activity in a temperature range from 40 °C to 60 °C and at pH range from 6.5 to 7.5. In general, lipases from *Aspergillus* species, as *A. niger*, present the highest activity between pH 4 and 7, and at temperature between 40 and 55 °C (Kamini et al., 1998; Namboodiri and Chattopadhyaya, 2000).

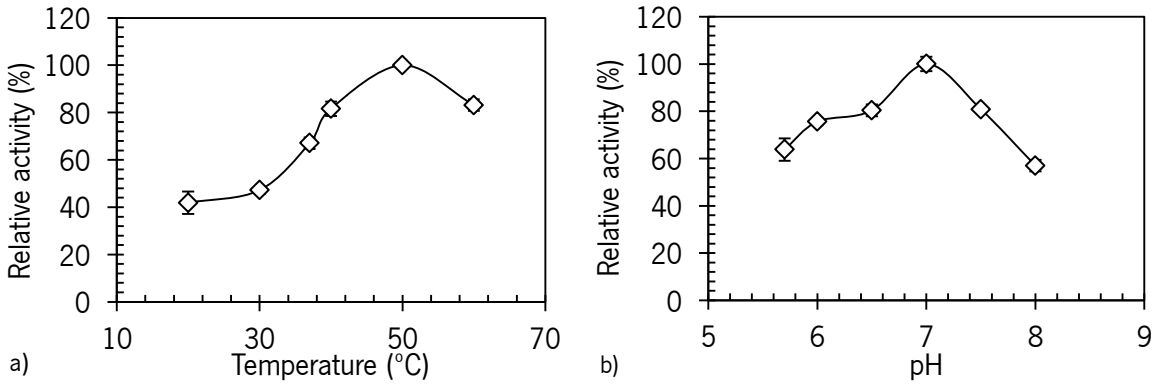

Figure 5.5 – Results of relative lipase activity as affected by the a) temperature at pH of 7, and by the b) pH at 37 °C, after 15 min of reaction time, in lipase sample. Values are the mean of triplicate analysis ± standard deviation.

Kamini et al. (1998) characterised *A. niger* lipase from SSF of gingelly oil cake and found maximum lipase activity at pH 7 and 37 °C. Mahadik et al. (2002) characterised *A. niger* lipase from SSF of WB with oil where the maximum lipase activity was found at 45 °C and pH of 2.5. Fleuri et al. (2014) studied lipases from *Aspergillus* sp. obtained from SSF of WB, soybean bran and soybean bran with sugarcane bagasse, and the maximum activity for the enzymes was at 50 °C but at different pH values. Similarly, Gutarra et al. (2009) found maximum activity at temperature of 50 °C, for *Penicillium simplicissimum* lipase by SSF of babassu cake, and differently, at pH 4.0 - 5.0, being an acidic lipase. Falony et al. (2006) reported an optimum pH of 6.0 and an optimum temperature of 40 °C, for lipase produced by *A. niger* by SSF of WB. Sun and Xu (2009) found optimum lipase activity at 40 °C and at pH 8, using *Rhizopus chinensis* lipase from SSF of WB and wheat flour.

As it can be seen, lipases characteristics may vary by the microorganism used, substrates used, and other conditions. *A. ibericus* lipase characteristics indicated the possible use on biochemical reactions at higher temperatures, and at neutral pH.

5.3.4.2 Time effect at room temperature

Results on Figure 5.6 show that the lipase from samples are stable during the first 3 days incubated at room temperature (around 20 °C), with maximum activity during the first 2 days and retaining around 80% of the activity on the 3th day. Similarly, Falony et al. (2006) reported that *A. niger* lipase from by SSF of WB retained almost 100% of the activity at 30 °C, after 24 h. And, Mahadik et al. (2002) found that *A. niger* lipase from SSF of WB with oil was stable at pH 7 retaining the activity, after 24 h at room temperature.

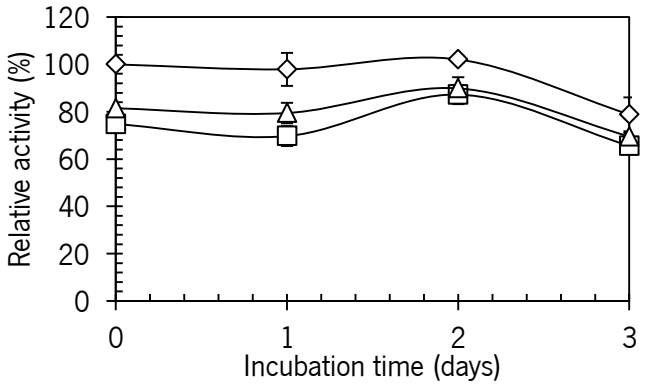

Figure 5.6 – Results of relative lipase activity over the days in (—◇—) lipase sample, (—□—) lipase lyophilised and ressuspended in buffer and (—△—) lipase lyophilised and ressuspended in water (in relation to lipase sample at time 0 days), exposed at room temperature. Values are the mean of triplicate analysis ± standard deviation.

Samples of lipase extract lyophilised and ressuspended in buffer and lipase lyophilised and ressuspended in water also presented a good enzyme stability over time, without statistical differences between them (Figure 5.6). A slight difference in relative activity was observed and an average of results from Figure 5.6 pointed a relative activity of 80 ± 7%, in relation to the lipase sample (liquid enzymatic extract) at different incubation times, showing that the lyophilisation is a

possible method to be implemented in order to preserve lipase and reduce volume space, with no significant losses in enzyme activity. A lipase activity of 1000 U per gram of lyophilised enzymatic extract was obtained.

5.3.5 Modifications in substrates composition after SSF

The substrates (OP and WB) were analysed before and after 7 days of SSF, to observe the nutritional composition modifications. Table 5.3 presents the main chemical parameters of these substrates, as follows: MC, ash, lignin (Lign), hemicellulose (HC), cellulose (Cell), lipids (Lip), protein concentration (PC), reducing sugars (RS), phenols (Phen), nitrogen (N), carbon (C), carbon/nitrogen ratio (C/N), crude protein (CP), and minerals and heavy metals. Figure 5.7a presents the respective increase or reduction in those characteristics after SSF, in relation to the initial characteristics of the substrate (mixture 1:1 of OP with WB).

The crude protein is an important parameter to consider in animal feed. Through SSF, the crude protein content increased in 81%, reaching 18% (w/w) in the dry solid fermented substrate. This increment is correlated to the fungal growth during SSF. Results confirmed the efficacy of the SSF to enhance the protein content of the fermented substrate (Li et al., 2013). Moreover, SSF improved the nutritional value in crude protein, in the range of the necessity for the animal feed to be used (Salgado et al., 2015). For example, a basal broiler chicken diets includes a crude protein level of 18.5% during the first 7 - 8 weeks and 16.0% for the subsequent 9 - 14 weeks (Li et al., 2013). Similarly to this work, Li et al. (2013) obtained a crude protein increase in 80%, using *A. niger* on SSF of solid wastes from *Silybum marianum* fruits. Salgado et al. (2015) also observed an increase in 107% using *A. uvarum* by SSF using mixture of winery by-products and OP. And, Canedo et al. (2016) found a 100% increase in crude protein content in the fermented wastes of brewery spent grain using *Rhizopus oligosporus*, after 7 days of SSF. The use of *A. ibericus* in the SSF enables to obtain a protein concentrate, which can be used as an alternative source for the use of by-products from olive mill industry for animal feed.

Table 5.3 – Characteristics of olive pomace (OP), wheat bran (WB) and fermented substrate (SSF OP+WB). Values are the mean of triplicate analysis ± standard deviation (SD).

Characteristics	Value ± SD			
	OP	WB	OP+WB	SSF (OP+WB)
MC (%, w/w)	73.5 ± 0.4	12.5 ± 0.1	58.2 ± 0.3	65 ± 2
Total solids (%, w/w)	26.5 ± 0.4	87.5 ± 0.1	41.8 ± 0.3	35 ± 2
Ash (g kg^{-1})	66 ± 5	62.4 ± 0.2	64 ± 3	22.6 ± 0.8
Lignin (g kg^{-1})	432 ± 5	29.7 ± 0.5	231 ± 3	332 ± 11
Hemicellulose (g kg^{-1})	223 ± 8	285 ± 8	254 ± 4	232 ± 1
Cellulose (g kg^{-1})	125 ± 9	290 ± 3	208 ± 6	160 ± 4
Lipids (g kg^{-1})	167 ± 1	36.6 ± 0.4	102 ± 1	24 ± 3
Protein (g kg^{-1})	4 ± 1	8.6 ± 0.2	6 ± 1	1.5 ± 0.3
Reducing sugars (g kg^{-1})	96 ± 6	15 ± 2	56 ± 4	17 ± 2
Phenols in water extract (g kg^{-1})	8.4 ± 0.3	3.8 ± 0.3	6.1 ± 0.3	4.9 ± 0.7
Phenols in MeOH extract (g kg^{-1})	11 ± 0.9	5 ± 0.1	8 ± 0.2	5.8 ± 0.6
Antioxidant activity (mmol Trolox kg^{-1})	55 ± 0.3	5.6 ± 0.7	30.6 ± 1.9	10.3 ± 0.3
N (%, w/w)	0.6 ± 0.1	2.57 ± 0.04	1.6 ± 0.1	2.87 ± 0.04
C (%, w/w)	49.7 ± 0.7	44.3 ± 0.2	47 ± 0.5	49.0 ± 0.2
C/N ratio	83 ± 7	17 ± 5	50 ± 6	17 ± 5
Crude protein (%, w/w)	3.8 ± 0.6	16.1 ± 0.3	9.9 ± 0.4	17.9 ± 0.3
Ca (g kg^{-1})	1.16 ± 0.04	1.85 ± 0.09	1.51 ± 0.07	2.3 ± 0.5
K (g kg^{-1})	17 ± 1	18 ± 1	18 ± 1	5.8 ± 0.5
Mg (g kg^{-1})	0.474 ± 0.22	5.1 ± 0.3	2.8 ± 0.3	2.9 ± 0.4
Zn (mg kg^{-1})	12 ± 0	145 ± 3	79 ± 2	96 ± 21
Cu (mg kg^{-1})	11.5 ± 0.7	14 ± 0	13 ± 0.4	17 ± 4
Fe (mg kg^{-1})	42 ± 2	134 ± 4	88 ± 3	103 ± 10
Mn (mg kg^{-1})	8.6 ± 0.1	128.5 ± 0.7	68.6 ± 0.4	74 ± 13
Cr (mg kg^{-1})	<22	<15	<19	<15
Ni (mg kg^{-1})	<22	<15	<19	<15
Pb (mg kg^{-1})	<22	<15	<19	<15
Na (mg kg^{-1})	373 ± 35	96 ± 1	235 ± 18	116 ± 17

Mass per total dry mass of substrates, exception for MC and total solids

After SSF, a reduction in 9% and 23% for hemicellulose and cellulose, respectively, was observed (Figure 5.7a). Lignocellulolytic enzymes as cellulases and xylanases showed a low activity, 5.1 ± 0.6 and 9.1 ± 0.5 U g^{-1}, respectively; and β-glucosidase presented an activity of 48 ±

1 U g^{-1} in the enzymatic extract. These enzymes caused the slight degradation of these components. These reductions may improve the digestibility and accessibility of these components by the digestive enzymes of animals (Salgado et al., 2015). In addition, the reduction of these components increased the concentration of lignin in the fermented substrate, since the presence of ligninases as lignin peroxidase, laccase and manganese peroxidase was not detected.

Phenolic compounds were reduced in 20% after 7 days of SSF by *A. ibericus*, even without the presence of laccase and peroxidases enzymes. Filamentous fungi are able to degrade phenolic compounds (García García et al., 2000). In previous work, the same strain of *A. ibericus* was used in SmF of diluted olive mill wastewater, where a reduction of 28% and 37% in phenolic compounds was observed (Abrunhosa et al., 2013). Similarly, Salgado et al. (2015) observed a reduction in 28% of phenolic compounds by *A. uvarum* after 10 days of SSF*,* using a mixture of winery by-products and OP. Reduction of phenolic compounds in the fermented substrate was positive, since they negatively affect the animal´s feed intake, feed digestibility and production efficiency (Salgado et al., 2015).

The antioxidant activity of unfermented and fermented substrates was determined (Table 5.3). It is noteworthy the high antioxidant activity of extracts from OP and low antioxidant activity of extracts from WB before SSF. After SSF, a reduction in antioxidant activity in fermented substrate was observed due to the extraction of phenolic compounds during enzymes recovery. The antioxidant activity in enzymatic extracts was determined achieving a 14.2 ± 1 mmol Trolox kg^{-1}, the sum of antioxidant activity in extract from fermented substrate and enzymatic extract was 24.5 mmol Trolox kg^{-1}. Thus, the antioxidant capacity was reduced after SSF. This may have been due to the reduction of phenolic compounds by fungus, as can be seen in methanolic extracts before and after SSF (Table 5.3). However, in the fermented substrate remained an interesting level of antioxidant activity. In the study of antioxidant activity in 112 medicinal plants was found a mean value of 9.4 mmol Trolox kg^{-1} in their methanolic extracts (Cai et al., 2004), this value was similar to the value observed in fermented substrate.

Lipids suffered a drastic reduction of 76%, after 7 days of SSF. This was a result of optimisation of SSF for lipase production. A lower final lipids concentration of 2.4 ± 0.3% was found. The obtained concentration is interesting for animal feed, since the lipids found in grains and forages naturally make-up approximately 2 - 4% of the ration (AgriAnalysis, 2016).

Figure 5.7b presents the modification in the percentage of minerals and heavy metals of the fermented substrate, in relation to the unfermented one. Highest reductions were found for potassium and sodium contents, and highest increases for calcium and copper. Results agreed with literature for copper, zinc, manganese and iron increases (Joshi and Sandhu, 1996; Salgado et al., 2015). Joshi and Sandhu (1996) reported the increase in those minerals after SSF of WB using different yeasts species, *Saccharomyces*, *Candida* and *Torula*. Also, Salgado et al. (2015) observed the increase of iron in the substrate by SSF of a mixture of winery by-products and OP using *A. uvarum*. The reduction of sodium was confirmed by Joshi and Sandhu (1996) and Salgado et al. (2015).

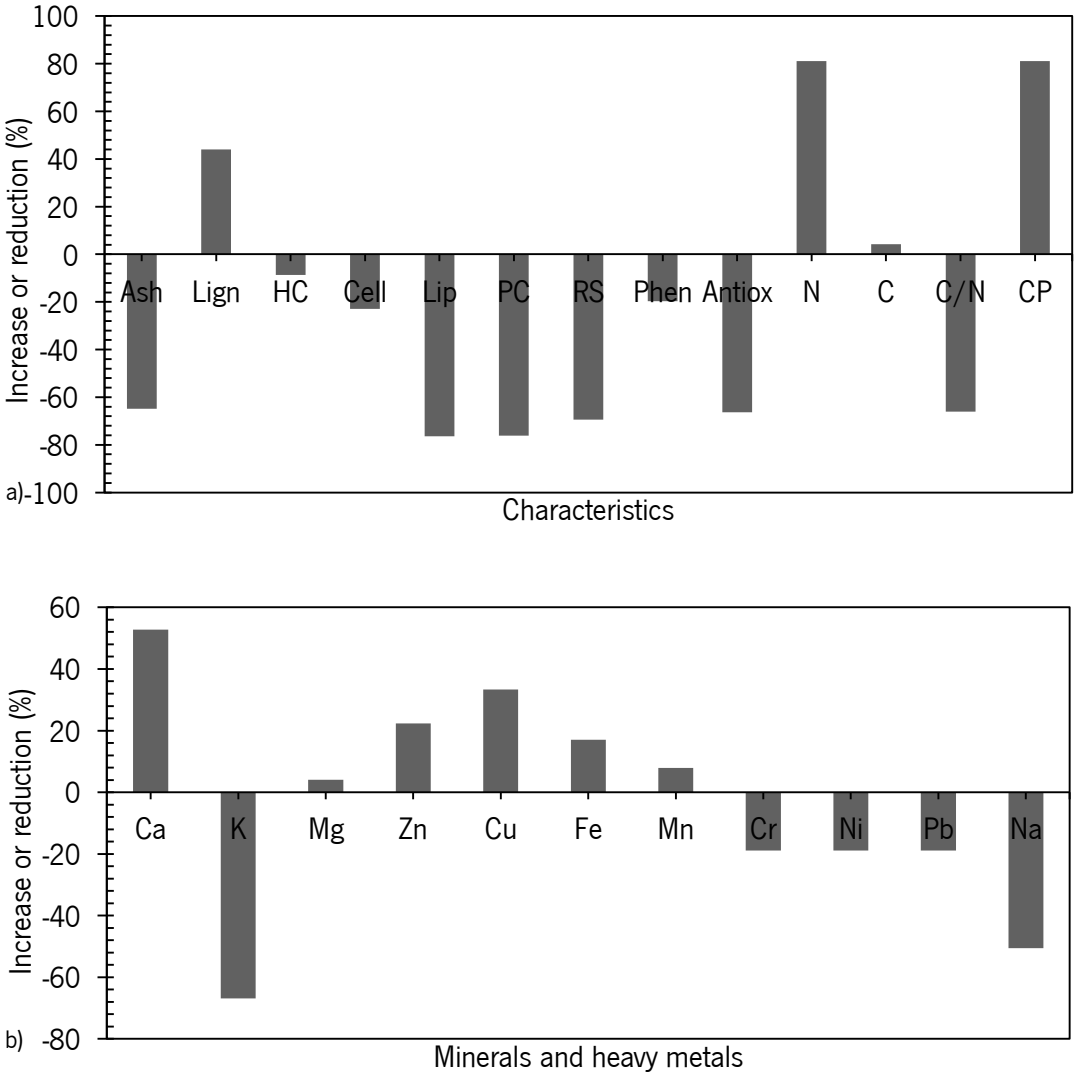

Figure 5.7 – Increase (positive values) or reduction (negative values) of a) the characteristics and b) minerals and heavy metals of the fermented substrate, after 7 days of SSF using sterilised substrate.

Contrary to the presented results, these authors found an increase in potassium and reduction in calcium and magnesium (Joshi and Sandhu, 1996; Salgado et al., 2015). An increase in microelements (calcium, magnesium, zinc, copper, iron and manganese) by fermentation had improved the nutritive value of OP with WB, with possible higher availability to livestock. In addition, it was observed the reduction of heavy metals contents (chromium, nickel and lead).

5.4 Conclusions

The effect of sterilisation of the substrate for SSF in small scale led to higher lipase production. However, it was proved that in large scale, the sterilisation step is not necessary. SSF was successfully 10-fold scaled-up to tray-type bioreactor but MC losses need to be prevented. For the first time ever reported SSF at pilot-scale pressure bioreactor was successfully used for lipase production. SSF scaling-up in this bioreactor seems to be more effective than in tray-type, improving specific lipase activity.

A. ibericus lipase showed maximum activity at 50 °C and pH 7.0. Its activity was retained for 3 days at room temperature. Lyophilisation of enzymatic extracts may be used as a way to preserve lipase, presenting an activity of 1000 U g^{-1} of lyophilised enzymatic extract. In addition, this work presented an integrated waste valorisation process by the increase of nutritional value of fermented substrate.

6 Optimisation of lipase production by *Aspergillus ibericus* from oil cakes and its application in esterification reactions

Due to the actual emphasis in revalorisation of agro-industrial residues and cost reduction of enzymes production, this work aimed the optimisation of lipase production from different oil cakes (OCs) produced in Brazil, by solid-state fermentation (SSF) using *Aspergillus ibericus* MUM 03.49 and the application of the produced lipase in hydrolysis and esterification reactions.

Higher lipase production was obtained using palm kernel oil cake (PKOC), yielding 127 ± 17 U g^{-1} of lipase (per mass of dry substrate). Through SSF optimisation, using PKOC mixed with sesame oil cake (SOC) in a ratio of 0.45 g of PKOC per g total substrate at 57% moisture content (MC), a production of 460 ± 38 U g^{-1} of lipase was obtained after 6 days of fermentation.

The obtained lipase was used in hydrolysis reactions, where it was observed higher activity in short-chain triacylglycerols (TAGs) substrates. Also, it was applied in esterification reactions, where the formation of butyl decanoate using 5% (w/v) of biocatalyst was the most efficient. SSF of PKOC and SOC is a low cost competitive process to obtain *A. ibericus* lipase that can be used in aroma esters production, with application in the food industry.

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6.1 Introduction

Oil cakes (OCs) are agro-industrial by-products obtained after oil extraction from seeds (Ramachandran et al., 2007). Their composition depend on their variety, growing conditions and extraction methods and can be classified as edible or non-edible oil cakes (Singhania et al., 2008). OCs are a good source of carbon, nitrogen and other compounds (Ramachandran et al., 2007). Thus, they are considered good substrates for fermentation processes as solid-state fermentation (SSF).

The last decade, increasing attention has been paid to the agro-industrial residues processing by SSF to obtain lipases with different applications (Couto and Sanromán, 2005), such as in the production of aroma esters, in a solvent-free system, which is of much commercial interest with the increasing demand of consumers for natural products (Ben Salah et al., 2007). Also, considering the scaling-up process, enzymatic esterification in a solvent-free system presents advantages, such as savings in reactor design for large-scale process and reduction in separation costs by avoiding solvent recovery costs (Bezbradica et al., 2007).

The aim of this work was to screen OCs from Brazil to optimise lipase production by *Aspergillus ibericus* MUM 03.49, and to study the effect of OCs composition on lipase production. The lipase produced was used in hydrolysis reactions to measure its activity in different substrates, triacylglycerols (TAGs) and olive oil; and also it was applied in enzymatic esterification reactions, in a solvent-free system, for the production of aroma esters.

6.2 Materials and methods

6.2.1 Microorganism

Aspergillus ibericus MUM 03.49 was used, as described in chapter 4.2.1.

6.2.2 OCs used as substrates

Several different oil cakes (OCs) were used. They were collected during the season 2013/2014 and were obtained from different suppliers, such as: Andiroba oil cake (AOC) and

cupuassu oil cake (CuOC) obtained from Beraca Ingredientes Naturais S.A. (São Paulo); canola oil cake (CaOC), macauba oil cake (MOC), palm kernel oil cake (PKOC) and soybean meal (SBM) from CENPES/PETROBRAS (Rio de Janeiro); crambe oil cake (CrOC), green coffee oil cake (GCOC) and sesame oil cake (SOC) from CRODA do Brasil, S.A. (Campinas – São Paulo). OCs were ground and sieved to provide a particle size ≤ 1.19 mm. The characteristics of the substrates were determined according to the literature (AOAC, 1995; IAL, 2005; Mendez et al., 1985; Van Soest, 1963). Moisture content (MC) was determined by drying the oil cake at 105 °C, ash by incineration of samples at 500 - 550 °C, and lipids content by Soxhlet method (AOAC, 1995; IAL, 2005). Protein content was determined by Micro-Kjeldahl method, using a factor of 6.25 to convert N to protein (AOAC, 1995). Carbohydrates nifext were determined by the subtraction of a hundred to the sum of MC, ash, lipids, protein and insoluble fibres, according to the legislation RCD 360/2003; and thus insoluble fibres content was not considered for the carbohydrates content. The determination of hemicellulose, cellulose and lignin was performed by determination of acid detergent fibre (ADF) and neutral detergent fibre (NDF) measurements, after extraction with acid detergent and neutral detergent, according to the procedures described by Van Soest (1963) and Mendez et al. (1985), respectively, using a six-plate setup for Dosi-fibre extraction (Tecnal). Lignin and cellulose was determined by using hydrolysis solution, according to Van Soest (1963), cellulose was determined by subtraction of lignin to ADF; and hemicellulose was determined by the subtraction of ADF (sum of cellulose and lignin) to NDF (sum of cellulose, hemicellulose and lignin). The analyses were performed in triplicate.

6.2.3 SSF of OCs for lipase production

SSFs were carried out using cotton-plugged 250 mL Erlenmeyer flasks containing 15 g of dry solid substrate. MC was adjusted to 60% (wet basis) by adding distilled water. Flasks were autoclaved at 121 °C for 15 min, cooled, inoculated with 0.5 mL spores suspension and incubated at 30 °C for 7 days. All fermentations were performed in duplicated flasks.

6.2.3.1 SSF with combination of OCs

Mixtures of the best OCs selected were used in SSF. PKOC was combined in a ratio of 1:1 (w/w) with CrOC and SOC. Fermentations were carried out using 7.5 g of each OC mixed with 7.5 g of PKOC, with the MC adjusted to 60%, keeping other conditions as previously described.

6.2.3.2 Influence of substrate ratio and MC on lipase production

The best mixture of OCs identified was further explored using an experimental design for optimisation of lipase production. The program Statistica 7 software (StatSoft, Tulsa, USA) was used to plan and analyse a central composite design, with two factors and two levels with additional star points. MC ranged from 50% to 70%, and the ratio of PKOC (RPKOC) in the substrate mixture (PKOC and SOC), expressed as g of PKOC per g of total substrate, ranged from 0.25 to 0.75 g g^{-1} of total substrate. The central point was performed in triplicate.

The relationship between the dependent (lipase activity) and independent (MC and RPKOC) variables was established by the polynomial Eq. (3.1), from chapter 3.2.4.

6.2.3.3 Supplementation with nitrogen source

SSFs were performed with addition of NH_4Cl, in order to supplement the substrates with a nitrogen source. Concentrations of NH_4Cl ranged from 0% to 5% (w/w) of NH_4Cl mass per dry mass of solid OC.

6.2.3.4 Time course profile of lipase production

Finally, a time course profile of lipase production and productivity was performed using the optimum SSF conditions determined in the optimisation experiment. Flasks were prepared as described before and destructively sampled each 2 days over a period of 20 days.

6.2.4 Enzymes extraction and determination

At the end of the incubation period, enzymes were extracted by adding 112.5 mL of 10 g L^{-1} Triton X-100 (7.5 mL g^{-1} dry solid substrate) to the fermented substrates and mixed at 250 rpm and 25 °C for 30 min using a shaker. Mixtures were then centrifuged (12000 × *g* for 10 min at 4 °C) and filtered using Whatman No. 1 filter paper. The resulting enzymatic extracts were immediately used for lipase and protease determination.

Lipase activity was determined by a spectrophotometric method, as described in chapter 4.2.5.

Protease was determined according to the method of Charney and Tomarelli (1947), using azocasein as the substrate. One unit of protease activity (U) was expressed as an increase in absorbance of 0.01 in relation to the blank per minute, under the assay conditions. The analyses were performed in duplicate. Protease activity was expressed as units per gram of dry solid substrate (U g^{-1}).

6.2.5 Lipase activity in different substrates and application in esterification reactions

6.2.5.1 Lipolytic activity in different substrates

Lipase produced was determined in several substrates. Olive oil and synthetic TAGs such as glyceryl tributyrate (C4:0), glyceryl trioctanoate (C8:0), glyceryl tridecanoate (C10:0), glyceryl tripalmitate (C16:0) and glyceryl triesterate (C18:0) in 0.01 M potassium phosphate buffer pH 7.0 were used. The hydrolysis reaction occurred at 37 °C for 20 min, at which time it was interrupted by the addition of 1:1 (v/v) acetone:ethanol mixture (1:1 v/v). Lipolytic activity was determined by titration of free fatty acids (FFAs) released with a pH-stat (916 Ti-Touch - Metrohm) using 0.04 M NaOH up to a final pH of 11, according to the methods of Freire et al. (1997) for olive oil, and Gutarra et al. (2009) for the synthetic TAGs. One unit of lipase activity (U) was expressed as the amount of enzyme which released 1 µmol of FFAs per minute, under the assay conditions. The analyses were performed in duplicate. Lipase activity obtained was expressed as units per gram of dry solid substrate (U g^{-1}).

6.2.5.2 Esterification reactions catalysed by lipase

Esterification reactions were performed using different organic acids and alcohols. The mixture used was a 1:1 molar ratio of organic acid to alcohol in a total volume of 5 mL. Lipase used was obtained from a 7 day SSF conducted under optimum conditions, with the fermented substrate containing the lipase simply being lyophilised. Reactions occurred in 15 mL falcon tubes with a 5 mL mixture of organic acid and alcohol in a 1:1 molar ratio, and 20% (w/v) of biocatalyst was added to the mixture. The Falcon tubes were incubated in a shaker at 200 rpm and 37 °C for 24 h, at which time 5 mL of 1:1 (v/v) acetone with absolute ethanol was added. Conversion (%) of the organic acid to ester was determined by titration of the remaining organic acid with 0.1 M NaOH until a pH equal to the pKa of the organic acid used. Esterification reactions were performed in duplicate.

Initially, different organic acids and alcohols were used. Combinations of lactic acid, butyric acid, propionic acid, hexanoic acid and decanoic acid with butanol were tested. Also, combinations of butyric acid with isobutanol, amyl alcohol, benzyl alcohol and menthol were tested.

Since decanoic acid with butanol presented the highest conversion (100%), this combination was used in further studies. The minimum lipase concentration needed to achieve 100% of conversion in 24 h was determined. For that, 20% (w/v), 10%, 5% and 1% of biocatalyst was used.

Using 10% (w/v) of biocatalyst, a time course profile of conversion over reaction time was obtained.

Finally, the effect of adding a buffer to the biocatalyst, in esterification reaction was studied. For that, a low amount of lyophilised biocatalyst, 5% (w/v), was added to Eppendorf tubes and ressuspended in 1 mL of Britton Robinson universal buffer (0.04 M H_3BO_3, 0.04 M H_3PO_4, 0.04 M CH_3COOH and 0.2 M NaOH) at pH values of 5, 6, 7, 8 and 9, and lyophilised again. The performance of this biocatalyst was subsequently analysed in the reaction of decanoic acid with butanol.

6.2.6 Statistical treatment

Principal components analyses (PCA) were conducted with Statgraphics Plus 5.1 (Manusgistics, Inc., Rockville, MD) to determine how the variation in the composition of the residues affected enzymes production.

Data obtained were statistically analysed using SPSS, as described in chapter 3.2.7.

6.3 Results and discussion

6.3.1 Characterisation of residues and evaluation of their effect on enzymes production

The characterisation of OCs and SBM are shown in Table 6.1. Significant differences of concentrations of protein, lipids, carbohydrates, and other compounds, were found between the different OCs used. These residues were used for SSF to produce lipase by *A. ibericus*. Lipase and protease produced after 7 days of SSF were significantly dependent ($p < 0.0001$) on the OCs used (Table 6.1). SSF using PKOC presented the highest lipase production following by SOC, but protease was not detected for PKOC, whereas for SOC a significant production was obtained.

In order to observe the relationship of residue components and enzyme production, PCA was performed involving the components of OCs that can affect the production of enzymes (ashes, proteins, cellulose, hemicellulose, lignin, carbohydrates, lipids): lipase and protease. This descriptive statistic tool allows grouping the original variables into fewer variables (principal components) that were used to carry out the representation. In this way, PCA allows to observe the main differences between the 9 agro-industrial residues and their effect on lipase and protease production.

The 3 first principal components (PC) contained 87% of the total variation in results, indicating the close interrelation among OCs characteristics and enzymes production. The first principal component (PC1) described 48% of the original variance, the second principal component (PC2) explained 23% and the third principal component (PC3) explained 16% of the variation.

Table 6.1 – Characteristics of oil cakes (mass per mass wet solid) and results of lipase activity and protease activity obtained from SSF of oil cakes. Values of characteristics are the mean of triplicate analysis ± standard deviation (SD); and values of lipase and protease are the mean of two individual fermentations ± standard deviation (SD). Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

Oil cakes	Moisture content ± SD (%, w/w)	Ash ± SD (%, w/w)	Protein content ± SD (%, w/w)	Lipids content ± SD (%, w/w)	Carbohydrates nifext ± SD (%, w/w)*	Hemicellulose ± SD (%, w/w)	Cellulose ± SD (%, w/w)	Lignin ± SD (%, w/w)	Lipase activity ± SD (U g^{-1})	Protease activity ± SD (U g^{-1})
AOC	7.3 ± 0.1	4.2 ± 0.1	7.9 ± 0.6	20.0 ± 0.6	17.8 ± 1.6	5.1 ± 1.4	15.3 ± 1.1	22.4 ± 1.1	1 ± 0^{a}	nd
CaOC	7.6 ± 0.2	5.2 ± 0.1	39.5 ± 0.9	11.9 ± 0.1	17.0 ± 0.7	3.4 ± 0.9	7.9 ± 0.4	7.5 ± 0.7	47 ± 11^{c}	35 ± 8^{c}
CrOC	8.1 ± 0.1	5.7 ± 0	28.1 ± 0.5	24.7 ± 0.1	11.8 ± 0	3.6 ± 0.6	9.6 ± 0.5	8.4 ± 0.6	44 ± 3^{bc}	33 ± 3^{bc}
CuOC	4.7 ± 0.2	7.2 ± 0.1	14.9 ± 0.5	21.3 ± 0.4	22.0 ± 0.3	6.3 ± 0.9	13.0 ± 1.0	10.7 ± 0.9	11 ± 1^{a}	7 ± 0^{a}
GCOC	4.1 ± 0.1	4.3 ± 0.1	17.8 ± 0.7	7.2 ± 0.2	9.9 ± 0.6	32.3 ± 0.8	21.7 ± 0.6	2.7 ± 0.7	4 ± 0^{a}	19 ± 1^{ab}
MOC	10.6 ± 0.1	4.0 ± 0.1	0.6 ± 0	13.2 ± 0.2	14.7 ± 1.8	19.4 ± 1.2	19.6 ± 0.5	17.9 ± 1.6	1 ± 0^{a}	7 ± 1^{a}
PKOC	7.9 ± 0.1	3.9 ± 0.1	1.2 ± 0.1	8.4 ± 0.1	3.6 ± 2.4	37.1 ± 0.9	18.0 ± 0.1	20.0 ± 1.8	127 ± 17^{e}	nd
SOC	4.0 ± 0.1	7.4 ± 0.1	37.1 ± 1.1	29.1 ± 0.1	2.0 ± 0.8	14.7 ± 0.1	4.6 ± 0.2	1.0 ± 0.1	78 ± 2^{d}	29 ± 6^{bc}
SBM	11.1 ± 0.2	6.1 ± 0	51.9 ± 1.5	1.4 ± 0.1	21.9 ± 1.4	2.6 ± 0.1	4.7 ± 0.2	0.3 ± 0	19 ± 0^{ab}	31 ± 2^{bc}

*Carbohydrates nifext – insoluble fibres content was not considered

Figure 6.1 shows the biplot representation of variables and scores of OCs and SBM on the plane defined by PC1 and PC2. PC1 is well correlated positively with cellulose, lignin, hemicellulose and negatively with ashes, proteins and protease activity. This PC1 grouped the residues with a lignocellulosic nature, such as PKOC, GCOC, MOC and AOC, on the right side. In addition, PC1 grouped the residues with high protein content, such as SBM and SOC, on the left side. Thus, the residues with higher fibres content showed a lower protein content. Protease was correlated with the concentration of protein in OCs (Figure 6.1). Since PKOC presents lower protein content, protease activity obtained was not detected, thus, in this substrate, the lipases formed are not degraded by proteases.

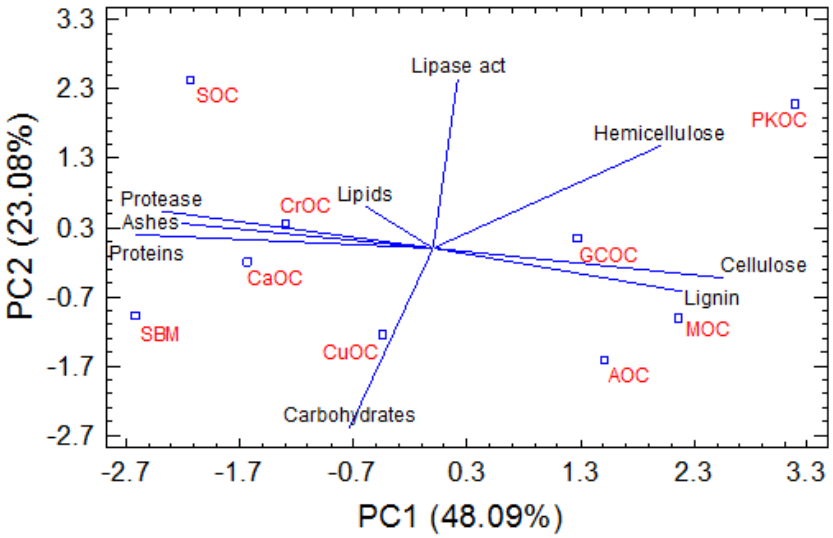

Figure 6.1 – Biplot representation of variables and scores of the oil cakes (OCs), defined by first principal component (PC1) and second principal component (PC2).

PC2 correlated well positively with lipase activity and negatively with carbohydrates. The OCs that achieved maximum lipase activity were PKOC and SOC that can be observed at the top of Figure 6.1. The negative effect of carbohydrates on lipase production was clear. The inhibitory effect of carbohydrates on lipase production was also observed by Arora et al. (2015). The presence of carbohydrates can induce the production of other enzymes as exoglucanases (Hanif et al., 2004) and limit the production of lipases. In addition, glucose presents a repressing effect on lipase production (Dalmau et al., 2000). The lipids content had a lower weigh on PC2 showing a

positive effect on lipase production. Some authors concluded that oil content increases lipase production (Gombert et al., 1999), as observed using SOC. However, the maximum lipase production was achieved by PKOC which has lower lipid content than other OCs, such as SOC. High lipids content above certain limits could inhibit the lipase production. In this sense, Damaso et al. (2008) observed a reduction of lipase production by *A. niger* with increasing olive oil addition to wheat bran (WB), and at 12% of olive oil lipase activity was no longer detected. Chen et al. (1998) observed a cell lysis in *Acinetobacter radioresistens* at high concentrations of olive oil that reduced the lipase production.

CaOC (*Brassica napus*) and CrOC (*Crambe abyssinica*) are together on the left side of the plot (Figure 6.1). These residues are generated after oil extraction from seed plants that are members of the same plant family (*Brassicaceae*), thus they have a similar composition. The same effect was observed between MOC and AOC. Both residues are obtained from seed oil extraction from Brazilian trees *Carapa guianensis* (Andiroba) and *Acrocomia aculeata* (Macaúba). The SBM is an outlier of the figure, insomuch as it is a substrate different to OCs, containing vestigial residual oil, high protein content and not being of lignocellulosic nature.

6.3.2 Study of synergic effect of OCs on lipase production

After screening OCs for lipase production by SSF, the residues that achieved a higher production were mixed to improve lipase activity. Mixtures of PKOC with two OCs, CrOC and SOC, were performed. Combinations of two OCs influenced significantly ($p < 0.01$) lipase production, where the use of PKOC+SOC led to a maximum production of 328 ± 6 U g^{-1}, corresponding to a 2.6-fold increase, compared to the value obtained on PKOC alone. Also, the mixture PKOC+CrOC presented improved yields, reaching 272 ± 23 U g^{-1}. The mixture of residues is attractive for the growth of microorganisms on SSF, since each residue may act differently as support matrix, nutrient source and as inducer for the production of enzymes (Edwinoliver et al., 2010). As can be observed, PKOC+SOC combination produced a synergic effect and improved lipase production. This effect may be due to an increase in the relative percentage of C18:n fatty acids (Lakshmi et al., 1999). Palm kernel oil has a low content of C18:n fatty acids, while sesame oil has a higher content in C18:n fatty acids (Ribeiro et al., 2016). Also, the mixture of both substrates may be

supplemented and diluted by each other with the protein, ashes and lipids content from SOC and, with the hemicellulose from PKOC, improving the lipase production.

Using the best combination of OCs, an optimisation study of MC and composition of PKOC and SOC in the substrate (RPKOC) mixture was performed to improve lipase yields. A central composite design was used and results are presented in Table 6.2. Lipase production was significantly affected ($p < 0.05$) by MC and RPKOC (Figure 6.2a). In fact, high or low levels of MC would significantly affect the biosynthesis of the enzymes (Sun and Xu, 2008). Low MC levels reduce the solubility of nutrients contained in solid substrate (Mahadik et al., 2002; Pandey, 2003; Sun and Xu, 2008), resulting in hampered microbial growth (Pandey, 2003; Singhania et al., 2009). High MC levels decrease substrate porosity, reduces gas volume and exchange, leading to oxygen diffusion limitation in the substrate layer (Edwinoliver et al., 2010; Hamidi-Esfahani et al., 2004; Singhania et al., 2009; Sun and Xu, 2008) and the microbial growth decreases (Hamidi-Esfahani et al., 2004).

Table 6.2 – Factors (moisture content (MC) and PKOC ratio (RPKOC), expressed as g of PKOC per g of total substrate), and assigned levels in a central composite design. Experimental and predicted values of lipase activity (LA) obtained. Experimental LA values are the mean of triplicate analysis ± standard deviation (SD).

Run	MC (g g^{-1})	RPKOC (g g^{-1})	Experimental LA ± SD (U g^{-1})	Predicted LA (U g^{-1})
1	0.5	0.25	313 ± 16	283
2	0.5	0.75	122 ± 22	145
3	0.7	0.25	55 ± 7	54
4	0.7	0.75	69 ± 25	121
5	0.4586	0.5	208 ± 31	217
6	0.7414	0.5	69 ± 37	38
7	0.6	0.1464	172 ± 27	199
8	0.6	0.8536	198 ± 16	149
9	0.6	0.5	377 ± 31	348
10	0.6	0.5	330 ± 15	348
11	0.6	0.5	336 ± 11	348

A polynomial equation, with the moisture content (MC) and the palm kernel oil cake ratio (RPKOC) as its independent variables, was fitted to experimental values of lipase activity (LA), as follows:

$$LA = -2929.5 + 11547.0\ MC - 11007.0\ MC^2 + 85.4\ RPKOC - 1387.7\ RPKOC^2 + 2052.3\ MC\ RPKOC \quad (6.1)$$

The analysis of variance indicated a satisfactory fitting of the equation to the experimental data, presenting a coefficient of determination (R^2) of 0.932. The variation of LA with MC and RPKOC was represented as response surface plot, using Statistica software (Figure 6.2b). To set optimum SSF conditions, the Solver tool of Excel was used to maximise lipase production as given by Eq. (6.1). A MC of 0.57 (57%) and a RPKOC of 0.45 g g^{-1} were obtained, predicting a lipase production of 360 U g^{-1}. Optimal conditions found were similar to MC and RPKOC used in previous experiments, leading to 10% improvement of lipase production. The result of optimum MC achieved was also similar to that found in chapter 3, using *A. ibericus* to produce lipase by SSF of olive pomace (OP) with WB.

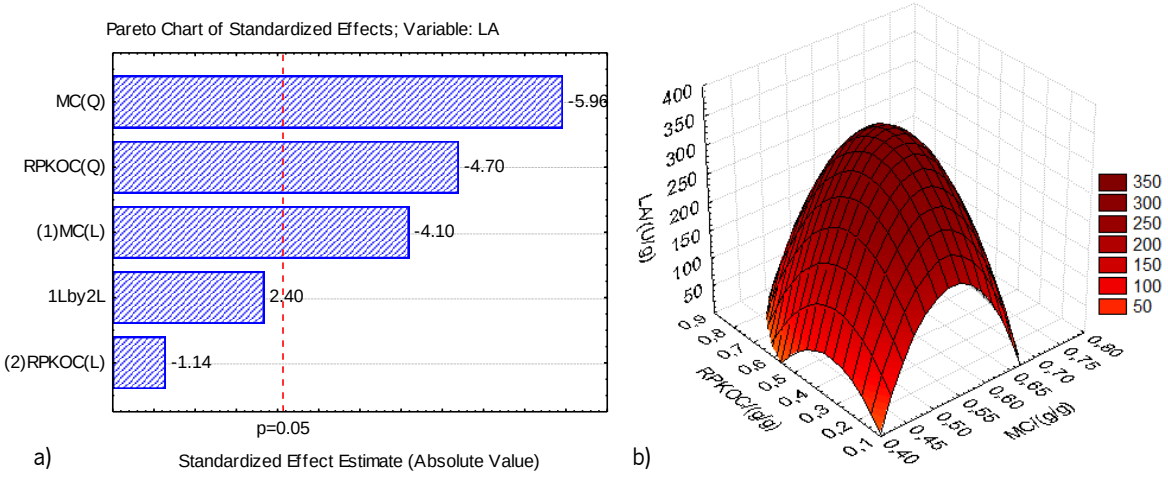

Figure 6.2 – a) Pareto chart of standardised effects of independent variables linear (L) and quadratic (Q), moisture content (MC) and PKOC ratio (RPKOC), expressed as g of PKOC per g of total substrate, on lipase activity (LA). b) Response surface of LA as a function of MC and RPKOC, according to Eq. (6.1).

For certain substrates, the supplementation with a nitrogen source may be need to enhance lipase production, thus the effect of NH_4Cl supplementation was studied. Increasing the NH_4Cl level from 0 to 1% (w/w) did not affect lipase production significantly (Tukey test), in spite of the 22% increase obtained at 1% (Figure 6.3). Thus, in further experiments 1% NH_4Cl was added to the

substrates for SSF. At higher levels of NH_4Cl (above 3%), the production was reduced (statistically significant), due to the possible inhibitory effect of NH_4Cl, as observed for other nitrogen sources by Imandi et al. (2013) and in chapter 4, using urea and $(NH_4)_2SO_4$, respectively.

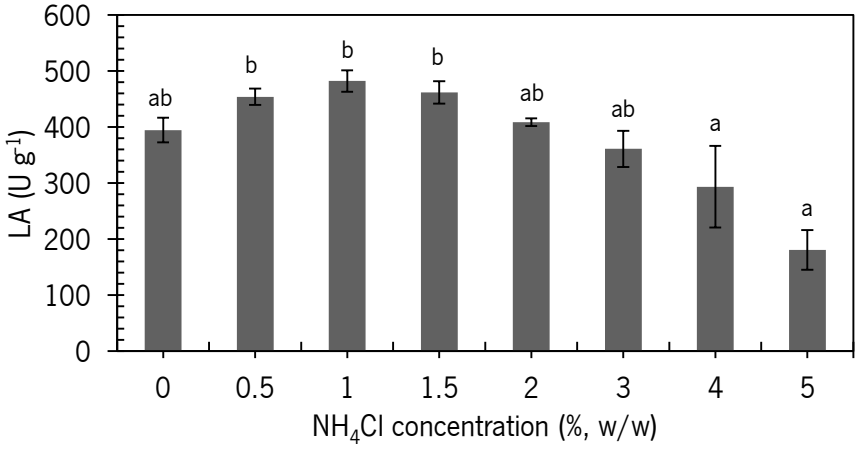

Figure 6.3 – Results of lipase activity (LA) influenced by the NH_4Cl concentration. Values are the mean of two individual fermentations ± standard deviation. Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

Finally, a time course profile of lipase production and its productivity was performed (Figure 6.4a). After 6 days, a lipase production of 460 ± 38 U g^{-1} was obtained, and it was increasing till 20 days of fermentation, yielding 578 ± 20 U g^{-1}. However, maximum productivity was obtained on the 6th day (3.2 ± 0.3 U g^{-1} h^{-1}). Contrary to lipase, protease activity reached its maximum of 45 ± 1 U g^{-1} on the 6th day and decreased to 28 ± 3 U g^{-1}, after 20 days of fermentation (Figure 6.4b).

The time course profiles of lipase and productivity were similar to the obtained in chapter 4, using *A. ibericus* on OP with WB. However, some authors found maximum lipase and productivity in a shorter time period, performing SSF at Erlenmeyer flasks level. Kamini et al. (1998) found a maximum lipase on the 3rd day, achieving 364 U g^{-1} and productivity of 5.1 U g^{-1} h^{-1} of *A. niger* lipase by SSF of gingelly oil cake. Similarly, Mala et al. (2007) obtained a maximum lipase on the 3rd day, achieving 384 U g^{-1} and productivity of 5.3 U g^{-1} h^{-1} of *A. niger* lipase by SSF of gingelly oil cake mixed with WB, observing an increment with the mixture of substrates. Edwinoliver et al. (2010) observed that SSF combining three substrates, wheat rawa, coconut oil cake and WB, led to maximum production of 629 U g^{-1} on the 4th day, and a productivity of 6.6 U g^{-1} h^{-1}. Mahadik et al.

(2002) obtained up to 300 U g^{-1} on the 5th day and a productivity up to 2.5 U g^{-1} h^{-1}, using *A. niger* by SSF of WB.

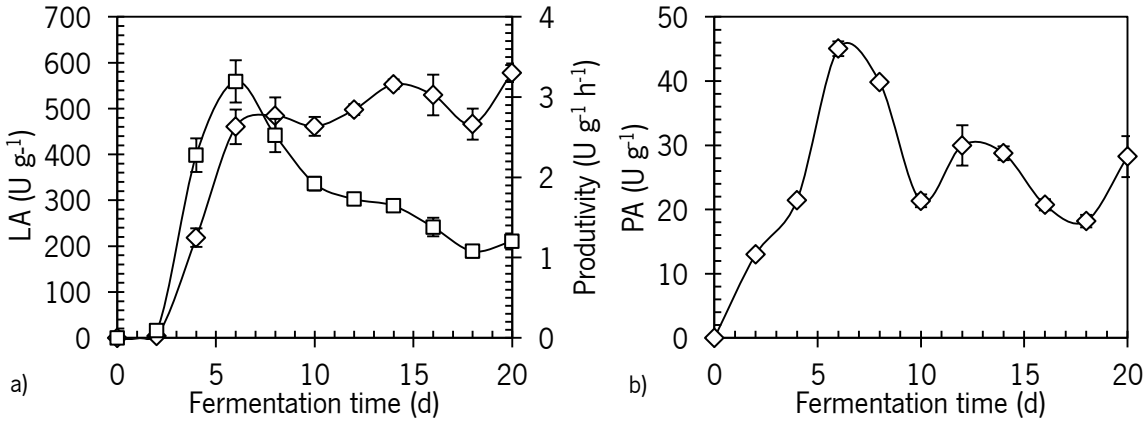

Figure 6.4 – Profiles of a) (—◇—) lipase activity (LA), (—□—) lipase productivity and b) protease activity (PA) over fermentation time. Values are the mean of two individual fermentations ± standard deviation.

This work presents an interesting study of the influence of OCs composition on lipase production, and showed the great importance of performing SSF using two selected OCs based on its composition, at optimum MC to improve lipase production, in agreement with the literature (Pandey, 2003; Pandey et al., 2000; Singhania et al., 2009) and previous work presented in chapter 3 and chapter 4. In addition, the use of PKOC with SOC doubled *A. ibericus* lipase production, comparing to the obtained lipase using OP with WB (chapter 4).

6.3.3 Lipase activity in different substrates and application in esterification reactions

6.3.3.1 Lipolytic activity tested in different substrates

The lipase produced at optimum SSF conditions was applied in hydrolysis reactions with different substrates. Figure 6.5 presents the results of lipase activity for each TAG used. Lipase was able to hydrolyse all TAGs, with maximum activity on C4:0 and C8:0, reaching 58 ±2 U g^{-1} and 61 ± 3 U g^{-1}, respectively. *A. ibericus* lipase showed higher activity for short-chain than for long-chain fatty acid esters, revealing high esterase activity (< 8 carbon atoms of chain fatty acid). An

intermediate lipase activity (40 ± 1 U g^{-1}) was observed using olive oil as substrate (Figure 6.5). Olive oil is a mixture of TAGs with long-chain fatty acids (> 95%): around 72% oleic acid (18:1), 14% palmitic acid (16:0), 9% linoleic acid (18:2) and 3% stearic acid (18:0) (Kamal-Eldin and Andersson, 1997), evidencing activity of the lipase for natural long-chain fatty acids, as it was produced in SSF of substrates containing a relatively high percentage of C18:n fatty acids, mainly from SOC. Lipolytic activity for synthetic C16:0 and C18:0 was very low. Similarly, Gutarra et al. (2009) found the highest lipolytic activities for C8:0 and C10:0, intermediate activities for olive oil and C16:0, and very low for C4:0 and C18:0, using *Penicillium simplicissimum* in SSF of babassu cake. These results of lipase activity obtained by titrimetric method using TAGs as substrates were significantly lower than that obtained by the spectrophotometric method using *p*-nitrophenyl butyrate (pNPB).

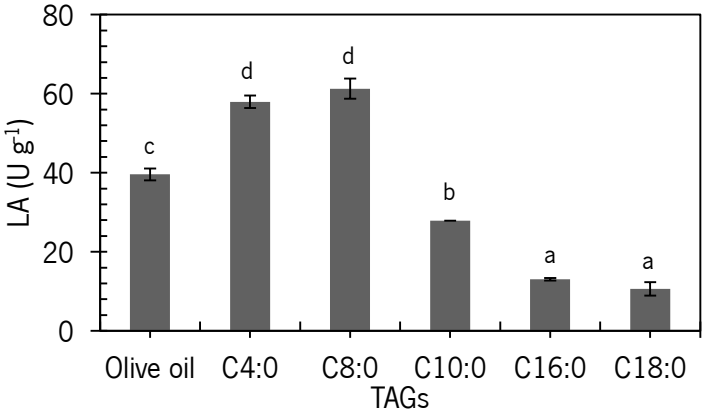

Figure 6.5 – Lipase activity (LA) determined by titration method, using different triacylglycerols (TAGs) as substrates. Values are the mean of duplicate analyses ± standard deviation. Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

6.3.3.2 Esterification reactions catalysed by lipase

Lipase with high stability in organic solvents may allow its use in esterification reactions (Gilham and Lehner, 2005). Strategies to improve kinetics and yields of esterification reactions catalysed by lipases may be achieved (Villeneuve, 2007). The optimisation of reaction conditions such as substrate used, enzyme load, reaction time and buffering may increase the lipase-catalysed production of aroma esters. Table 6.3 presents the conversion of organic acids to esters

in the esterification reaction combining different organic acids and alcohols. Maximum conversion (100%) was observed for the combination of decanoic acid with butanol, leading to the production of butyl decanoate aroma ester. Also, butyric acid with menthol led to high conversion (93 ± 11%).

Table 6.3 – Conversion of esterification reactions using different organic acids and alcohols, using 20% (w/v) biocatalyst from SSF of PKOC+SOC, after 24 h of reaction at 200 rpm and 37 °C. Values are the mean of two individual reactions ± standard deviation (SD). Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

Esterification reaction	Conversion ± SD (%)
Lactic acid + butanol	0^{a}
Butyric acid + butanol	28 ± 8^{bc}
Propionic acid + butanol	9 ± 4^{ab}
Hexanoic acid + butanol	55 ± 0^{d}
Decanoic acid + butanol	100 ± 0^{e}
Butyric acid + isobutanol	29 ± 0^{bc}
Butyric acid + amyl alcohol	46 ± 11^{cd}
Butyric acid + benzyl alcohol	16 ± 0^{ab}
Butyric acid + menthol	93 ± 11^{e}

The polarity of the reaction mixture may affect lipase activity. Substrates with high polarity may inhibit enzyme activity (Sun et al., 2013), by damaging the water micro-layer in a vicinity of the lipase, which is necessary to keep the enzyme in its active conformation (Ben Salah et al., 2007; Bezbradica et al., 2007). The lower conversions using lactic acid, butyric acid and propionic acid were due to the high polarity of these substrates (Table 6.4).

Table 6.4 – Literature values of Log *P* of individual organic acids and alcohols.

Organic acid	Log *P*	Alcohol	Log *P*
Lactic acid	-0.72	Butanol	0.84
Butyric acid	0.79	Isobutanol	0.79
Propionic acid	0.33	Amyl alcohol	1.10
Hexanoic acid	1.92	Benzyl alcohol	1.10
Decanoic acid	4.09	Menthol	3.38

On the contrary, using decanoic acid with butanol and butyric acid with menthol, the conversion was higher since higher log *P* values of decanoic acid and menthol contributed to the decreased polarity of the reaction mixture (Sun et al., 2013).

Log *P* is defined as the logarithm of the partition coefficient of a substrate in the standard 1-octanol-water two-phase system (Dutra et al., 2008). The use of non-polar substrates for enzymatic esterification may increase conversion, by increasing log *P* of the reaction mixture, as determined in numerous studies (Bezbradica et al., 2007; Vermue and Tramper, 1995). A linear correlation between log *P* of the reaction mixture (Table 6.4) and conversion (Table 6.3) was found, with a satisfactory coefficient of determination (R^2) of 0.9355 (Figure 6.6). Therefore, the mixture of decanoic acid with butanol in esterification reactions was used in further studies.

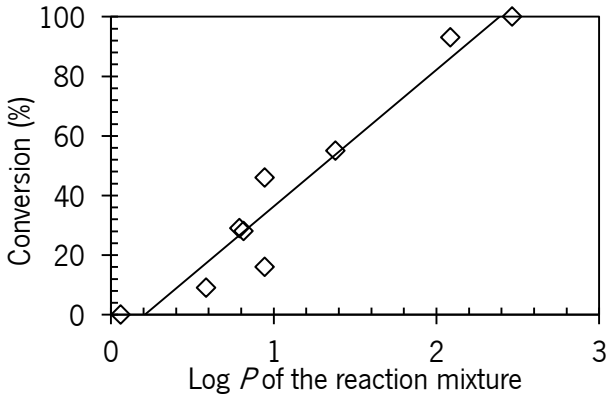

Figure 6.6 – Results of conversion (Table 6.3) as a function of log *P* of the reaction mixture, and representation of a linear regression fitted to the experimental values.

From an economic perspective, achieving high conversion utilising a low amount of enzyme in the reaction is important. Therefore, the effect of the biocatalyst load on esterification reaction of decanoic acid with butanol, for a 24 h reaction time, was studied. At least 10% (w/v) of biocatalyst was needed to obtain a maximum conversion (100%) (Table 6.5a). The use of 5% (w/v) of biocatalyst led to 5-fold decrease in the conversion. Based on these results, a biocatalyst load of 10% (w/v) was used in further experiments.

Table 6.5 – Conversion in esterification reactions of decanoic acid with butanol at 200 rpm and 37 °C, a) using different biocatalyst load, after 24 h of reaction; b) over reaction time using 10% (w/v) biocatalyst; and c) using 5% (w/v) biocatalyst lyophilised in universal buffer at different pH, after 24 h of reaction. Values are the mean of two individual reactions ± standard deviation (SD). Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test).

Conditions/ Conversion ± SD (%)						
a) Biocatalyst load (%, w/v)	1	5	10	20	-	-
Conversion ± SD (%)	2 ± 1^{a}	22 ± 1^{b}	100 ± 0^{c}	100 ± 0^{c}	-	-
b) Reaction time (h)	0	2	4	8	16	24
Conversion ± SD (%)	0^{a}	28 ± 11^{b}	25 ± 7^{b}	58 ± 4^{c}	68 ± 4^{c}	100 ± 0^{d}
c) Universal buffer pH	5	6	7	8	9	-
Conversion ± SD (%)	64 ± 6^{a}	71 ± 6^{ab}	100 ± 0^{c}	100 ± 0^{c}	82 ± 2^{b}	-

A time course profile of conversion over reaction time was performed, using 10% (w/v) of biocatalyst in the mixture of 5 mL 1:1 (molar) of decanoic acid with butanol. As presented in Table 6.5b, the conversion increased gradually reaching maximum conversion (100%) after 24 h reaction.

Since lyophilisation of the enzyme together with salts has been shown to greatly improve the enzyme performance (Triantafyllou et al., 1997), leading to higher conversion, thus in these esterification reactions only 5% of the biocatalyst was used and it was possible to obtain the maximum conversion (100%) with universal buffer at pH of 7 and 8 (Table 6.5c). Salts may induce activation of lipase due to the polar environment constituted by the salt-bonded water surrounding the lipase, increasing enzyme flexibility (Jin et al., 2013). Also, the buffer pH used during the lyophilisation of the biocatalyst seemed important. Zaks and Klibanov (1985) observed that the catalytic activity of lipase in the organic medium very much depends on the pH from which the enzyme was precipitated, with the maximum approximately coinciding with the optimum pH of the enzymatic activity in water. This effect is due to the fact that ionogenic groups of the enzyme acquire a certain ionisation state in the aqueous solution of a given pH. This ionisation state (and the enzymatic activity corresponding to it) is retained in the solid state and in organic solvents (Zaks and Klibanov, 1988).

A. ibericus lipase was able to produce butyl decanoate ester. This aroma ester has relevant interest for food industry. Butyl decanoate ester is a colourless liquid with a characteristic odour of whiskey, and can be applied to products requiring a dairy product/creamy note, with a dosage of 7

- 35 ppm in the finished product (Unitedint, 2016). The optimisation process led to obtain maximum conversion, using 5% (w/v) of biocatalyst lyophilised in universal buffer at pH 7 or 8 for 24 h in the esterification reaction of decanoic acid with butanol, in a solvent-free system.

6.4 Conclusions

Agro-industrial residues and its combination used for SSF by *A. ibericus* led to different lipase yields. Mixing PKOC with SOC is a promising strategy to obtain high lipase yields while treating wastes. Optimising SSF conditions, a final lipase production of 460 ± 38 U g^{-1}, and maximum productivity of 3.2 ± 0.3 U g^{-1} h^{-1} were obtained after 6 days of fermentation. Lipase produced was effective to produce butyl decanoate ester in a solvent-free system, at optimum esterification reaction conditions.

This work presents an environmental friendly strategy to naturally produce an aroma ester in a solvent-free system, with application in the food industry, while adding value to the agro-industrial residues.

7 General conclusions and final remarks

This chapter presents the overall conclusions and the main outcomes of this Ph.D. thesis.

This chapter also presents the suggestions for future work, based on the results obtained.

7.1 General conclusions

This work provides an environmental friendly global strategy to the agro-industrial residues, as OCs, in particular OP. OCs can be biotechnologically valorised obtaining an added-value compound, as lipase, with high yields, produced by a filamentous fungus. After screening stages, *A. ibericus*, a new isolated fungus, was identified as the best lipase producer under SSF conditions.

Important parameters with high impact on biotechnology processes as MC and temperature were optimised, achieving a maximum lipase production with 60% MC and 30 °C. On the other hand, the lipase production was improved by mixing OP with WB, other low cost substrate, in ratio of 1:1 (w,w, dry basis). In addition, due to the MC of these substrates, it was not necessary to set the MC by addition of water, thus they were used directly without pre-treatment allowing the reduction of costs.

The optimised conditions were successfully tested in a lab-scale packed-bed bioreactor. Other parameters as aeration rate and recovery of lipase after SSF were optimised. An aeration flow of 0.05 L min^{-1} improved the lipase production. Furthermore, the addition of Triton X-100 increased the lipase extraction achieving a lipase activity of 223 U g^{-1} dry solid substrate (OP+WB) on 7th day of SSF.

Lipase production by SSF in OP with WB was successfully scaled-up, using 300 - 500 g of dry substrate. For the first time, a novel pilot-scale continuous pressurised bioreactor was used for lipase production by SSF. This bioreactor proved to be more efficient than traditional tray-type bioreactor, at 200 kPa.

A. ibericus was tested in other OCs. The statistical analysis of OCs composition and enzymes production allowed to predict the lipase production according to OCs characteristics. All OCs studied, the mixture of PKOC with SOC achieved the highest lipase yields. Optimising SSF conditions, a final lipase production of 460 U g^{-1} of dry solid substrate (PKOC+SOC) on the 6th day was observed.

The final products of the SSF process were characterised. Lipase extracts showed maximum activity at 50 °C and pH 7.0. After lyophilisation stage, lipase powder presented an activity of 1000 U g^{-1}, which may be used in industrial processes. In addition, the fermented substrate was characterised, where it was observed an increase of nutritional value that may be applied as animal feed. Lipase obtained from OCs was further applied in esterification reactions, and it was effective

to produce butyl decanoate ester in a solvent-free system, giving an application of lipase to food industry.

7.2 Suggestions for future work

In spite of the present work bring new insights on lipase production by SSF, and optimisation of parameters of relevance for the bioprocess; there are still some new issues for future study.

The study at tray-type bioreactor was preliminary, and it would be very interesting to fully understand the role of parameters such as substrate bed height, temperature, film covering, continuous control of humidity, use of perforated trays with aeration and its control, etc., in order to achieve a relationship between these variables, and to optimise lipase. Also, the use of a rotary-drum bioreactor in this process would be interesting to compare with the bioreactors type used in this work, optimising operating factors such as aeration rate and rotary velocity.

The study of the impact of pre-treatments on lipase production by SSF of the residues mixtures used in this work could allow to further improve the growth and fungi activity due to the liberation of more easily degradable sugars. Pre-treatments such as heat, microwaves, ultrasounds and chemical hydrolysis could be tested.

A preliminary study on lipase applications was performed, as in esterification reactions for aroma ester production, as presented in chapter 6. It would be interesting to apply lipase produced in other reactions, as for biodiesel production, in detergents, etc. Also, to purify the lipase and perform a molecular and structural characterisation.

Lipase immobilisation on low-cost materials and applicability of the biocatalyst obtained is also an important issue to study that may enable the lipase commercialisation.

The fermented substrate characterised for animal feed, needs further evaluation, as to analyse the palate and acceptability by animals.

Finally, an economic analysis of the all process will be necessary to prove the all concept, including substrates availability and cost, industrial equipment and operating costs, as well as the biocatalyst possible prize and the price for the final fermented solid as an additive for animal feed formulations.

8 References

This chapter lists all the references which contributed to the elaboration of this Ph.D. thesis.

Abrunhosa, L., Oliveira, F., Dantas, D., Gonçalves, C., Belo, I., 2013. Lipase production by *Aspergillus ibericus* using olive mill wastewater. Bioprocess Biosyst. Eng. 36, 285–91.

AgriAnalysis, 2016. Feed and Forage Terminology from Agri Analysis, Inc. http://www.agrianalysis.com/feed-and-forage-terminology.shtml (accessed 19.10.16).

Aijun, Z., Hongzhang, C., Zuohu, L., 2005. Air pressure pulsation solid state production of alkaline protease by *Bacillus pumilus* 1.1625. Process Biochem. 40, 1547–1551.

Alburquerque, J.A., González, J., García, D., Cegarra, J., 2004. Agrochemical characterisation of "alperujo", a solid by-product of the two-phase centrifugation method for olive oil extraction. Bioresour. Technol. 91, 195–200.

Alburquerque, J.A., Gonzálvez, J., García, D., Cegarra, J., 2006a. Measuring detoxification and maturity in compost made from "alperujo", the solid by-product of extracting olive oil by the two-phase centrifugation system. Chemosphere 64, 470–477.

Alburquerque, J.A., Gonzálvez, J., García, D., Cegarra, J., 2006b. Effects of bulking agent on the composting of "alperujo", the solid by-product of the two-phase centrifugation method for olive oil extraction. Process Biochem. 41, 127–132.

Aliakbarian, B., Casazza, A. a., Perego, P., 2011. Valorization of olive oil solid waste using high pressure-high temperature reactor. Food Chem. 128, 704–710.

Amin, M., Bhatti, H.N., 2014. Effect of physicochemical parameters on lipase production by *Penicillium fellutanum* using canola seed oil cake as substrate. Int. J. Agric. Biol. 16, 118–124.

AOAC, 1995. Official methods of analysis of AOAC international, 16th ed, Association of Official Analytical Chemists.

Apprich, S., Tirpanalan, Ö., Johannes, H., Reisinger, M., Böhmdorfer, S., Siebenhandl-Ehn, S., Novalin, S., Kneifel, W., 2014. Wheat bran-based biorefinery 2: valorization of products. LWT - Food Sci. Technol. 56, 222–231.

Arora, M., Patel, J., Baser, I., Chhasatia, M.R., 2015. Effect of medium constituents on the growth and lipase production in *Pseudomonas aeruginosa* 2036. Int. J. Adv. Res. Biol. Sci. 2, 171–176.

Arvanitoyannis, I.S., Kassaveti, A., 2007. Current and potential uses of composted olive oil waste.

Int. J. Food Sci. Technol. 42, 281–295.

Atimtay, A.T., Topal, H., 2004. Co-combustion of olive cake with lignite coal in a circulating fluidized bed. Fuel 83, 859–867.

Ávila-Cisneros, N., Velasco-Lozano, S., Huerta-Ochoa, S., Córdova-López, J., Gimeno, M., Favela-Torres, E., 2014. Production of thermostable lipase by *Thermomyces lanuginosus* on solid-state fermentation: selective hydrolysis of sardine oil. Appl. Biochem. Biotechnol. 174, 1859–1872.

Azbar, N., Bayram, A., Filibeli, A., Muezzinoglu, A., Sengul, F., Ozer, A., 2004. A review of waste management options in olive oil production. Crit. Rev. Environ. Sci. Technol. 34, 209–247.

Balaji, V., Ebenezer, P., 2008. Optimization of extracellular lipase production in *Colletotrichum gloeosporioides* by solid state fermentation. Indian J. Sci. Technol. 1, 1–8.

Ballesteros, I., Oliva, J.M., Saez, F., Ballesteros, M., 2001. Ethanol production from lignocellulosic byproducts of olive oil extraction. Appl. Biochem. Biotechnol. 91–93, 237–252.

Bano, Z., Shashirekha, M.N., Rajarathnam, S., 1993. Improvement of the bioconversion and biotransformation efficiencies of the oyster mushroom (*Pleurotus sajor-caju*) by supplementation of its rice straw substrate with oil seed cakes. Enzyme Microb. Technol. 15, 985–989.

Basheer, S.M., Chellappan, S., Beena, P.S., Sukumaran, R.K., Elyas, K.K., Chandrasekaran, M., 2011. Lipase from marine *Aspergillus awamori* BTMFW032: production, partial purification and application in oil effluent treatment. N. Biotechnol. 28, 627–38.

Bellon-Maurel, V., Orliac, O., Christen, P., 2003. Sensors and measurements in solid state fermentation: a review. Process Biochem. 38, 881–896.

Belo, I., Pinheiro, R., Mota, M., 2003. Fed-batch cultivation of *Saccharomyces cerevisiae* in a hyperbaric bioreactor. Biotechnol. Prog. 19, 665–671.

Belo, I., Pinheiro, R., Mota, M., 2005. Morphological and physiological changes in *Saccharomyces cerevisiae* by oxidative stress from hyperbaric air. J. Biotechnol. 115, 397–404.

Ben Salah, R., Ghamghui, H., Miled, N., Mejdoub, H., Gargouri, Y., 2007. Production of butyl acetate ester by lipase from novel strain of *Rhizopus oryzae*. J. Biosci. Bioeng. 103, 368–372.

Bezbradica, D., Mijin, D., Šiler-Marinković, S., Knežević, Z., 2007. The effect of substrate polarity on the lipase-catalyzed synthesis of aroma esters in solvent-free systems. J. Mol. Catal. B Enzym. 45, 97–101.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cai, Y., Luo, Q., Sun, M., Corke, H., 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 74, 2157–2184.

Canedo, M.S., de Paula, F.G., da Silva, F.A., Vendruscolo, F., 2016. Protein enrichment of brewery spent grain from *Rhizopus oligosporus* by solid-state fermentation. Bioprocess Biosyst. Eng. 39, 1105–1113.

Castilho, L.R., Polato, C.M.S., Baruque, E.A., Sant´Anna, J., Freire, D.M.G., 2000. Economic analysis of lipase production by *Penicillium restrictum* in solid-state and submerged fermentations. Biochem. Eng. J. 4, 239–247.

Charney, J., Tomarelli, R.M., 1947. A colorimetric method for the determination of the proteolytic activity of duodenal juice. J. Biol. Chem. 171, 501–505.

Chen, H.-Z., Zhao, Z.-M., Li, H.-Q., 2014. The effect of gas double-dynamic on mass distribution in solid-state fermentation. Enzyme Microb. Technol. 58–59, 14–21.

Chen, S.J., Cheng, C.Y., Chen, T.L., 1998. Production of an alkaline lipase by *Acinetobacter radioresistens*. J. Ferment. Bioeng. 86, 308–312.

Coca, J., Hernández, O., Berrio, R., Martínez, S., Díaz, E., Dustet, J.C., 2001. Production and characterization of lipases from *Aspergillus niger* and *Aspergillus fumigatus*. Biotecnol. Apl. 18, 216–220.

Colla, L.M., Rizzardi, J., Pinto, M.H., Reinehr, C.O., Bertolin, T.E., Costa, J.A.V., 2010. Simultaneous production of lipases and biosurfactants by submerged and solid-state bioprocesses. Bioresour. Technol. 101, 8308–8314.

Commenil, P., Belingheri, L., Sancholle, M., Dehorter, B., 1995. Purification and properties of an extracellular lipase from the fungus *Botrytis cinerea*. Lipids 30, 351–356.

Contesini, F.J., Lopes, D.B., Macedo, G.A., Nascimento, M.D.G., Carvalho, P.D.O., 2010. *Aspergillus* sp. lipase: Potential biocatalyst for industrial use. J. Mol. Catal. B Enzym. 67,

163–171.

Coradi, G.V., Da Visitação, V.L., De Lima, E.A., Saito, L.Y.T., Palmieri, D.A., Takita, M.A., De Oliva Neto, P., De Lima, V.M.G., 2013. Comparing submerged and solid-state fermentation of agro-industrial residues for the production and characterization of lipase by *Trichoderma harzianum*. Ann. Microbiol. 63, 533–540.

Couto, S.R., Sanromán, M.A., 2005. Application of solid-state fermentation to ligninolytic enzyme production. Biochem. Eng. J. 22, 211–219.

Couto, S.R., Sanromán, M.Á., 2006. Application of solid-state fermentation to food industry – a review. J. Food Eng. 76, 291–302.

Dalmau, E., Montesinos, J., Lotti, M., Casas, C., 2000. Effect of different carbon sources on lipase production by *Candida rugosa*. Enzyme Microb. Technol. 26, 657–663.

Damaso, M.C.T., Passianoto, M.A., de Freitas, S.C., Freire, D.M.G., Lago, R.C.A., Couri, S., 2008. Utilization of agroindustrial residues for lipase production by solid-state fermentation. Brazilian J. Microbiol. 39, 676–681.

Dermeche, S., Nadour, M., Larroche, C., Moulti-Mati, F., Michaud, P., 2013. Olive mill wastes: biochemical characterizations and valorization strategies. Process Biochem. 48, 1532–1552.

Díaz, A.B., Alvarado, O., de Ory, I., Caro, I., Blandino, A., 2013. Valorization of grape pomace and orange peels: improved production of hydrolytic enzymes for the clarification of orange juice. Food Bioprod. Process. 91, 580–586.

Díaz, A.B., Caro, I., de Ory, I., Blandino, A., 2007. Evaluation of the conditions for the extraction of hydrolitic enzymes obtained by solid state fermentation from grape pomace. Enzyme Microb. Technol. 41, 302–306.

Dobrev, G., Zhekova, B., Dobreva, V., Strinska, H., Doykina, P., Krastanov, A., 2015. Lipase biosynthesis by *Aspergillus carbonarius* in a nutrient medium containing products and byproducts from the oleochemical industry. Biocatal. Agric. Biotechnol. 4, 77–82.

Dulf, F.V., Vodnar, D.C., Dulf, E.H., Toşa, M.I., 2015. Total phenolic contents, antioxidant activities, and lipid fractions from berry pomaces obtained by solid-state fermentation of two *Sambucus* species with *Aspergillus niger*. J. Agric. Food Chem. 63, 3489–3500.

Dundar, A.N., Gocmen, D., 2013. Effects of autoclaving temperature and storing time on resistant

starch formation and its functional and physicochemical properties. Carbohydr. Polym. 97, 764–771.

Dutra, J.C. V, da C. Terzi, S., Bevilaqua, J.V., Damaso, M.C.T., Couri, S., Langone, M.A.P., Senna, L.F., 2008. Lipase production in solid-state fermentation monitoring biomass growth of *Aspergillus niger* using digital image processing. Appl. Biochem. Biotechnol. 147, 63–75.

Edwinoliver, N.G., Thirunavukarasu, K., Naidu, R.B., Gowthaman, M.K., Kambe, T.N., Kamini, N.R., 2010. Scale up of a novel tri-substrate fermentation for enhanced production of *Aspergillus niger* lipase for tallow hydrolysis. Bioresour. Technol. 101, 6791–6796.

El-Bakry, M., Gea, T., Sánchez, A., 2016. Inoculation effect of thermophilic microorganisms on protease production through solid-state fermentation under non-sterile conditions at lab and bench scale (SSF). Bioprocess Biosyst. Eng. 39, 585–592.

Falony, G., Armas, J.C., Mendoza, J.C.D., Hernández, J.L.M., 2006. Production of extracellular lipase from *Aspergillus niger* by solid-state fermentation. Food Technol. Biotechnol. 44, 235–240.

FAO, 2016. Food and Agriculture Organization of the United Nations. http://www.fao.org/faostat/en/#home (accessed 1.2.17).

Figueroa-Montero, A., Esparza-Isunza, T., Saucedo-Castañeda, G., Huerta-Ochoa, S., Gutiérrez-Rojas, M., Favela-Torres, E., 2011. Improvement of heat removal in solid-state fermentation tray bioreactors by forced air convection. J. Chem. Technol. Biotechnol. 86, 1321–1331.

Fleuri, L.F., Novelli, P.K., Delgado, C.H.O., Pivetta, M.R., Pereira, M.S., Arcuri, M. de L.C., Capoville, B.L., 2014. Biochemical characterisation and application of lipases produced by *Aspergillus* sp. on solid-state fermentation using three substrates. Int. J. Food Sci. Technol. 49, 2585–2591.

Freire, D.M., Teles, E.M., Bon, E.P., Sant' Anna, G.L., 1997. Lipase production by *Penicillium restrictum* in a bench-scale fermenter: effect of carbon and nitrogen nutrition, agitation, and aeration. Appl. Biochem. Biotechnol. 63–65, 409–421.

Galanakis, C.M., 2012. Recovery of high added-value components from food wastes: conventional, emerging technologies and commercialized applications. Trends Food Sci. Technol. 26, 68–87.

Ganguly, S., Nandi, S., 2015. Process optimization of lipase catalyzed synthesis of diesters in a packed bed reactor. Biochem. Eng. J. 102, 2–5.

García García, I., Jiménez Peña, P.R., Bonilla Venceslada, J.L., Martín Martín, A., Martín Santos, M.A., Ramos Gómez, E., 2000. Removal of phenol compounds from olive mill wastewater using *Phanerochaete chrysosporium*, *Aspergillus niger*, *Aspergillus terreus* and *Geotrichum candidum*. Process Biochem. 35, 751–758.

Gavala, H.N., Skiadas, I. V., Ahring, B.K., Lyberatos, G., 2005. Potential for biohydrogen and methane production from olive pulp. Water Sci. Technol. 52, 209–215.

Gilham, D., Lehner, R., 2005. Techniques to measure lipase and esterase activity in vitro. Methods 36, 139–147.

Gombert, A.K., Pinto, A.L., Castilho, L.R., Freire, D.M.G., 1999. Lipase production by *Penicillium restrictum* in solid-state fermentation using babassu oil cake as substrate. Process Biochem. 35, 85–90.

Gomes, N., Gonçalves, C., García-Román, M., Teixeira, J.A., Belo, I., 2011. Optimization of a colorimetric assay for yeast lipase activity in complex systems. Anal. Methods 3, 1008–1013.

Gutarra, M.L.E., Cavalcanti, E.D.C., Castilho, L.R., Freire, D.M.G., Jr., G.L.S., 2005. Lipase production by solid-state fermentation: cultivation conditions and operation of tray and packed-bed bioreactors. Appl. Biochem. Biotechnol. 121–124, 105–116.

Gutarra, M.L.E., Godoy, M.G., Maugeri, F., Rodrigues, M.I., Freire, D.M.G., Castilho, L.R., 2009. Production of an acidic and thermostable lipase of the mesophilic fungus *Penicillium simplicissimum* by solid-state fermentation. Bioresour. Technol. 100, 5249–5254.

Hamidi-Esfahani, Z., Shojaosadati, S.A., Rinzema, A., 2004. Modelling of simultaneous effect of moisture and temperature on *A. niger* growth in solid-state fermentation. Biochem. Eng. J. 21, 265–272.

Hanif, A., Yasmeen, A., Rajoka, M.I., 2004. Induction, production, repression, and de-repression of exoglucanase synthesis in *Aspergillus niger*. Bioresour. Technol. 94, 311–319.

Hardin, M.T., Mitchell, D.A., Howes, T., 2000. Approach to designing rotating drum bioreactors for solid-state fermentation on the basis of dimensionless design factors. Biotechnol. Bioeng. 67, 274–282.

Hasan, F., Shah, A.A., Hameed, A., 2006. Industrial applications of microbial lipases. Enzyme Microb. Technol. 39, 235–251.

Herrgard, S., Gibas, C.J., Subramaniam, S., 2000. Role of electrostatic network of residues in the enzymatic action of *Rhizomucor miehei* lipase family. Biochemistry 39, 2921–2930.

IAL, 2005. Métodos químicos e físicos para análise de alimentos, 4th ed, Normas Analíticas do Instituto Adolfo Lutz. São Paulo.

Ilmi, M., Hidayat, C., Hastuti, P., Heeres, H.J., van der Maarel, M.J.E.C., 2017. Utilisation of *Jatropha* press cake as substrate in biomass and lipase production from *Aspergillus niger* 65I6 and *Rhizomucor miehei* CBS 360.62. Biocatal. Agric. Biotechnol. 9, 103–107.

Imandi, S.B., Karanam, S.K., Garapati, H.R., 2010. Optimization of media constituents for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from palm kernal cake (*Elaeis guineensis*). Adv. Biosci. Biotechnol. 1, 115–121.

Imandi, S.B., Karanam, S.K., Garapati, H.R., 2013. Use of Plackett-Burman design for rapid screening of nitrogen and carbon sources for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from mustard oil cake (*Brassica napus*). Brazilian J. Microbiol. 44, 915–921.

INE, 2016. Instituto Nacional de Estatística. https://www.ine.pt/xportal/xmain?xpgid=ine_main&xpid=INE (accessed 1.2.17).

Jacobsen, T., Poulsen, O.M., 1995. Comparison of lipases from different strains of the fungus *Geotrichum candidum*. Biochim. Biophys. Acta 1257, 96–102.

Jia, J., Yang, X., Wu, Z., Zhang, Q., Lin, Z., Guo, H., Lin, C.S.K., Wang, J., Wang, Y., 2015. Optimization of fermentation medium for extracellular lipase production from *Aspergillus niger* using response surface methodology. Biomed Res. Int. 2015, 1–8.

Jin, Q., Jia, G., Wang, X., Li, C., 2013. Comparison of the salt-induced activation of *Pseudomonas cepacia* lipase in organic media by phosphate buffer and sodium sulfate. Chinese J. Catal. 34, 1224–1231.

Joseph, B., Ramteke, P.W., Thomas, G., 2008. Cold active microbial lipases: some hot issues and recent developments. Biotechnol. Adv. 26, 457–470.

Joshi, V.K., Sandhu, D.K., 1996. Preparation and evaluation of an animal feed byproduct produced

by solid-state fermentation of apple pomace. Bioresour. Technol. 56, 251–255.

Kamal-Eldin, A., Andersson, R., 1997. A multivariate study of the correlation between tocopherol content and fatty acid composition in vegetable oils. J. Am. Oil Chem. Soc. 74, 375–380.

Kamini, N.R., Mala, J.G.S., Puvanakrishnan, R., 1998. Lipase production from *Aspergillus niger* by solid-state fermentation using gingelly oil cake. Process Biochem. 33, 505–511.

Khan, T.A., Saxena, S.K., 1997. Integrated management of root knot nematode Meloidogyne javanica infecting tomato using organic materials and *Paecilomyces lilacinus*. Bioresour. Technol. 61, 247–250.

Kota, K., Sridhar, P., 1999. Solid state cultivation of *Streptomyces clavuligerus* for cephamycin C production. Process Biochem. 34, 325–328.

Kumar, S., Katiyar, N., Ingle, P., Negi, S., 2011. Use of evolutionary operation (EVOP) factorial design technique to develop a bioprocess using grease waste as a substrate for lipase production. Bioresour. Technol. 102, 4909–4912.

Lakshmi, B.S., Kangueane, P., Abraham, B., Pennathur, G., 1999. Effect of vegetable oils in the secretion of lipase from *Candida rugosa* (DSM 2031). Lett. Appl. Microbiol. 29, 66–70.

Leite, P., Salgado, J.M., Venâncio, A., Domínguez, J.M., Belo, I., 2016. Ultrasounds pretreatment of olive pomace to improve xylanase and cellulase production by solid-state fermentation. Bioresour. Technol. 214, 737–746.

Li, F., Li, F., Zhao, T., Mao, G., Zou, Y., Zheng, D., Takase, M., Feng, W., Wu, X., Yang, L., 2013. Solid-state fermentation of industrial solid wastes from the fruits of milk thistle *Silybum marianum* for feed quality improvement. Appl. Microbiol. Biotechnol. 97, 6725–6737.

Lima, V.M.G., Krieger, N., Mitchell, D.A., Fontana, J.D., 2004. Activity and stability of a crude lipase from *Penicillium aurantiogriseum* in aqueous media and organic solvents. Biochem. Eng. J. 18, 65–71.

Lima, V.M.G., Krieger, N., Sarquis, M.I.M., Mitchell, D.A., Ramos, L.P., Fontana, J.D., 2003. Effect of nitrogen and carbon sources on lipase production by *Penicillium aurantiogriseum*. Food Technol. Biotechnol. 41, 105–110.

Longo, M.A., Deive, F.J., Domínguez, A., Sanromán, M.A., 2008. Solid-state fermentation for food and feed application, in: Pandey, A., Soccol, C.R., Larroche, C. (Eds.), Current developments

in solid-state fermentation. Springer, New Delhi, pp. 379–411.

Lonsane, B.K., Ghildyal, N.P., Budiatman, S., Ramakrishna, S.V., 1985. Engineering aspects of solid state fermentation. Enzyme Microb. Technol. 7, 258–265.

Lopes, M., Gomes, N., Gonçalves, C., Coelho, M.A.Z., Mota, M., Belo, I., 2008. *Yarrowia lipolytica* lipase production enhanced by increased air pressure. Lett. Appl. Microbiol. 46, 255–260.

Lopes, M., Gomes, N., Mota, M., Belo, I., 2009. *Yarrowia lipolytica* growth under increased air pressure: influence on enzyme production. Appl. Biochem. Biotechnol. 159, 46–53.

Lopes, V.R.O., Farias, M.A., Belo, I.M.P., Coelho, M.A.Z., 2016. Nitrogen sources on TPOMW valorization through solid state fermentation performed by *Yarrowia lipolytica*. Brazilian J. Chem. Eng. 33, 261–270.

Lu, M., Brooks, J.D., Maddox, I.S., 1997. Citric acid production by solid-state fermentation in a packed-bed reactor using *Aspergillus niger*. Enzyme Microb. Technol. 21, 392–397.

Lu, M.Y., Maddox, I.S., Brooks, J.D., 1998. Application of a multi-layer packed-bed reactor to citric acid production in solid-state fermentation using *Aspergillus niger*. Process Biochem. 33, 117–123.

Lu, W., Li, D., Wu, Y., 2003. Influence of water activity and temperature on xylanase biosynthesis in pilot-scale solid-state fermentation by *Aspergillus sulphureus*. Enzyme Microb. Technol. 32, 305–311.

Mahadik, N.D., Puntambekar, U.S., Bastawde, K.B., Khire, J.M., Gokhale, D. V, 2002. Production of acidic lipase by *Aspergillus niger* in solid state fermentation. Process Biochem. 38, 715–721.

Mala, J.G.S., Edwinoliver, N.G., Kamini, N.R., Puvanakrishnan, R., 2007. Mixed substrate solid state fermentation for production and extraction of lipase from *Aspergillus niger* MTCC 2594. J. Gen. Appl. Microbiol. 53, 247–253.

Mendez, M.H.M., Derivi, S.C.N., Rodrigues, M.C.R., Fernandes, M.L., Machado, R.L.D., 1985. Método de fibra detergente neutro modificado para amostras ricas em amido. Ciência e Tecnol. Aliment. 5, 123–131.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.

Miranda, O.A., Salgueiro, A.A., Pimentel, M.C.B., Filho, J.L.L., Melo, E.H.M., Durán, N., 2000. Lipase production by a Brazilian strain of *Penicillium citrinum* using an industrial residue. Bioresour. Technol. 69, 145–147.

Mitchell, D.A., Krieger, N., Stuart, D.M., Pandey, A., 2000. New developments in solid-state fermentation II. Rational approaches to the design, operation and scale-up of bioreactors. Process Biochem. 35, 1211–1225.

Molla, A.H., Fakhru'l-Razi, A., Hanafi, M.M., Alam, M.Z., 2004. Optimization of process factors for solid-state bioconversion of domestic wastewater sludge. Int. Biodeterior. Biodegrad. 53, 49–55.

Namboodiri, V.M., Chattopadhyaya, R., 2000. Purification and biochemical characterization of a novel thermostable lipase from *Aspergillus niger*. Lipids 35, 495–502.

Ohtsuki, T., Akiyama, J., Shimoyama, T., Yazaki, S.-I., Ui, S., Hirose, Y., Mimura, A., 2003. Increased production of antioxidative sesaminol glucosides from sesame oil cake through fermentation by *Bacillus circulans* strain YUS-2. Biosci. Biotechnol. Biochem. 67, 2304–2306.

Oliveira, F., Moreira, C., Salgado, J.M., Abrunhosa, L., Venâncio, A., Belo, I., 2016. Olive pomace valorization by *Aspergillus* species: lipase production using solid-state fermentation. J. Sci. Food Agric. 96, 3583–3589.

Pal, A., Khanum, F., 2010. Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. Bioresour. Technol. 101, 7563–7569.

Palma, M.B., Annette, L.P., Gombert, A.K., Seitz, K.H., Kivatinitz, S.C., Castilho, L.R., Freire, D.M.G., 2000. Lipase production by *Penicillium restrictum* using solid waste of industrial babassu oil production as substrate. Appl. Biochem. Biotechnol. 84–86, 1137–1145.

Pandey, A., 2003. Solid-state fermentation. Biochem. Eng. J. 13, 81–84.

Pandey, A., Soccol, C.R., Mitchell, D., 2000. New developments in solid state fermentation: I-bioprocesses and products. Process Biochem. 35, 1153–1169.

Pensupa, N., Jin, M., Kokolski, M., Archer, D.B., Du, C., 2013. A solid state fungal fermentation-based strategy for the hydrolysis of wheat straw. Bioresour. Technol. 149, 261–267.

Pérez-Rodríguez, N., Oliveira, F., Pérez-Bibbins, B., Belo, I., Torrado Agrasar, A., Domínguez, J.M.,

2014. Optimization of xylanase production by filamentous fungi in solid-state fermentation and scale-up to horizontal tube bioreactor. Appl. Biochem. Biotechnol. 173, 803–825.

Pimentel, M. do C.B., Melo, E.H.M., Filho, J.L.L., Ledingham, W.M., Durán, N., 1997. Lipase from a Brazilian strain *Penicillium citrinum* cultured in a simple and inexpensive medium: heat-denaturation, kinetics, and pH stability. Appl. Biochem. Biotechnol. 66, 185–195.

Raghavarao, K.S.M.S., Ranganathan, T.V., Karanth, N.G., 2003. Some engineering aspects of solid-state fermentation. Biochem. Eng. J. 13, 127–135.

Rajagopalan, S., Modak, J.M., 1994. Heat and mass transfer simulation studies for solid-state fermentation processes. Chem. Eng. Sci. 49, 2187–2193.

Rajendran, A., Thangavelu, V., 2009. Statistical experimental design for evaluation of medium components for lipase production by *Rhizopus arrhizus* MTCC 2233. LWT - Food Sci. Technol. 42, 985–992.

Ramachandran, S., Singh, S.K., Larroche, C., Soccol, C.R., Pandey, A., 2007. Oil cakes and their biotechnological applications - a review. Bioresour. Technol. 98, 2000–2009.

Ramos-Cormenzana, A., Monteoliva-Sánchez, M., López, M.J., 1995. Bioremediation of alpechin. Int. Biodeterior. Biodegradation 35, 249–268.

Reis, P., Holmberg, K., Watzke, H., Leser, M.E., Miller, R., 2009. Lipases at interfaces: a review. Adv. Colloid Interface Sci. 147–148, 237–250.

Ribeiro, S.A.O., Nicacio, A.E., Zanqui, A.B., Biondo, P.B.F., de Abreu-Filho, B.A., Visentainer, J.V., Gomes, S.T.M., Matsushita, M., 2016. Improvements in the quality of sesame oil obtained by a green extraction method using enzymes. LWT - Food Sci. Technol. 65, 464–470.

Rigo, E., Ninow, J.L., Polloni, A.E., Remonatto, D., Arbter, F., Vardanega, R., de Oliveira, D., Treichel, H., Di Luccio, M., 2009. Improved lipase biosynthesis by a newly isolated *Penicillium* sp. grown on agricultural wastes. Ind. Biotechnol. 5, 119–126.

Rodriguez, J.A., Mateos, J.C., Nungaray, J., González, V., Bhagnagar, T., Roussos, S., Cordova, J., Baratti, J., 2006. Improving lipase production by nutrient source modification using *Rhizopus homothallicus* cultured in solid state fermentation. Process Biochem. 41, 2264–2269.

Roig, A., Cayuela, M.L., Sánchez-Monedero, M.A., 2006. An overview on olive mill wastes and their valorisation methods. Waste Manag. 26, 960–969.

Romdhane, I.B. Ben, Fendri, A., Gargouri, Y., Gargouri, A., Belghith, H., 2010. A novel thermoactive and alkaline lipase from *Talaromyces thermophilus* fungus for use in laundry detergents. Biochem. Eng. J. 53, 112–120.

Ruiz, B., Farrés, A., Langleya, E., Massoc, F., Sáncheza, S., 2001. Purification and characterization of an extracellular lipase from *Penicillium candidum*. Lipids 36, 283–289.

Salgado, J.M., Abrunhosa, L., Venâncio, A., Domínguez, J.M., Belo, I., 2014a. Screening of winery and olive mill wastes for lignocellulolytic enzyme production from *Aspergillus* species by solid-state fermentation. Biomass Convers. Biorefinery 4, 201–209.

Salgado, J.M., Abrunhosa, L., Venâncio, A., Domínguez, J.M., Belo, I., 2014b. Integrated use of residues from olive mill and winery for lipase production by solid state fermentation with *Aspergillus* sp. Appl. Biochem. Biotechnol. 172, 1832–1845.

Salgado, J.M., Abrunhosa, L., Venâncio, A., Domínguez, J.M., Belo, I., 2015. Enhancing the bioconversion of winery and olive mill waste mixtures into lignocellulolytic enzymes and animal feed by *Aspergillus uvarum* using a packed-bed bioreactor. J. Agric. Food Chem. 63, 9306–9314.

Salihu, A., Alam, M.Z., AbdulKarim, M.I., Salleh, H.M., 2012. Lipase production: an insight in the utilization of renewable agricultural residues. Resour. Conserv. Recycl. 58, 36–44.

Saxena, R.K., Sheoran, A., Giri, B., Davidson, W.S., 2003. Purification strategies for microbial lipases. J. Microbiol. Methods 52, 1–18.

Serra, R., Cabañes, F.J., Perrone, G., Castellá, G., Venâncio, A., Mulè, G., Kozakiewicz, Z., 2006. *Aspergillus ibericus*: a new species of section *Nigri* isolated from grapes. Mycologia 98, 295–306.

Sidebottom, C.M., Charton, E., Dunn, P.P.J., Mycock, G., Davies, C., Sutton, J.L., Macrae, A.R., Slabas, A.R., 1991. *Geotrichum candidum* produces several lipases with markedly different substrate specificities. Eur. J. Biochem. 202, 485–491.

Silveira, E.A., Tardioli, P.W., Farinas, C.S., 2016. Valorization of palm oil industrial waste as feedstock for lipase production. Appl. Biochem. Biotechnol. 179, 558–571.

Singhania, R.R., Patel, A.K., Soccol, C.R., Pandey, A., 2009. Recent advances in solid-state fermentation. Biochem. Eng. J. 44, 13–18.

Singhania, R.R., Soccol, C.R., Pandey, A., 2008. Application of tropical agro-industrial residues as substrate for solid-state fermentation processes, in: Pandey, A., Soccol, C.R., Larroche, C. (Eds.), Current developments in solid-state fermentation. Springer, New Delhi, pp. 412–442.

Sircar, A., Sridhar, P., Das, P.K., 1998. Optimization of solid state medium for the production of clavulanic acid by *Streptomyces clavuligerus*. Process Biochem. 33, 283–289.

Stuart, D.M., Mitchell, D. A., Johns, M.R., Litster, J.D., 1999. Solid-state fermentation in rotating drum bioreactors: operating variables affect performance through their effects on transport phenomena. Biotechnol. Bioeng. 63, 383–391.

Sun, J., Yu, B., Curran, P., Liu, S.-Q., 2013. Lipase-catalysed ester synthesis in solvent-free oil system: Is it esterification or transesterification? Food Chem. 141, 2828–2832.

Sun, S.Y., Xu, Y., 2008. Solid-state fermentation for "whole-cell synthetic lipase" production from *Rhizopus chinensis* and identification of the functional enzyme. Process Biochem. 43, 219–224.

Sun, S.Y., Xu, Y., 2009. Membrane-bound "synthetic lipase" specifically cultured under solid-state fermentation and submerged fermentation by *Rhizopus chinensis*: a comparative investigation. Bioresour. Technol. 100, 1336–1342.

Tengerdy, R.P., Szakacs, G., 2003. Bioconversion of lignocellulose in solid substrate fermentation. Biochem. Eng. J. 13, 169–179.

Toida, J., Arikawa, Y., Kondou, K., Fukuzawa, M., Sekiguchi, J., 1998. Purification and characterization of triacylglycerol lipase from *Aspergillus oryzae*. Biosci. Biotechnol. Biochem. 62, 759–763.

Triantafyllou, A.O., Wehtje, E., Adlercreutz, P., Mattiasson, B., 1997. How do additives effect enzyme activity and stability in non-aqueous media? Biotechnol. Bioeng. 54, 67–76.

Ülker, S., Karaoglu, S.A., 2012. Purification and characterization of an extracellular lipase from *Mucor hiemalis* f. *corticola* isolated from soil. J. Biosci. Bioeng. 114, 385–390.

Unitedint, 2016. United International Inc. http://www.unitedint.com/en/proshow.asp?id=405&classid=41 (accessed 4.12.16).

Van Soest, P.J., 1963. Use of detergent in the analysis of fibrous feed I, preparation of fiber residues of low nitrogen. J. Assoc. Off. Agric. Chem. 46, 925–929.

Vaseghi, Z., Najafpour, G.D., Mohseni, S., Mahjoub, S., 2013. Production of active lipase by *Rhizopus oryzae* from sugarcane bagasse: solid state fermentation in a tray bioreactor. Int. J. Food Sci. Technol. 48, 283–289.

Venkatesagowda, B., Ponugupaty, E., Barbosa, A.M., Dekker, R.F.H., 2015. Solid-state fermentation of coconut kernel-cake as substrate for the production of lipases by the coconut kernel-associated fungus *Lasiodiplodia theobromae* VBE-1. Ann. Microbiol. 65, 129–142.

Vermue, M.H., Tramper, J., 1995. Biocatalysis in non-conventional media: medium engineering aspects. Pure Appl. Chem. 67, 345–373.

Villeneuve, P., 2007. Lipases in lipophilization reactions. Biotechnol. Adv. 25, 515–536.

Viniegra-González, G., Favela-Torres, E., Aguilar, C.N., Rómero-Gomez, S.D.J., Díaz-Godínez, G., Augur, C., 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochem. Eng. J. 13, 157–167.

Virupakshi, S., Babu, K.G., Gaikwad, S.R., Naik, G.R., 2005. Production of a xylanolytic enzyme by a thermoalkaliphilic *Bacillus* sp. JB-99 in solid state fermentation. Process Biochem. 40, 431–435.

Wang, D., Xu, Y., Shan, T., 2008. Effects of oils and oil-related substrates on the synthetic activity of membrane-bound lipase from *Rhizopus chinensis* and optimization of the lipase fermentation media. Biochem. Eng. J. 41, 30–37.

Xu, F.J., Chen, H.Z., Li, Z.H., 2002. New developments in engineering aspects of solid-state fermentation. Prog. Biotechnol. 22, 44–48.

Zaks, A., Klibanov, A.M., 1985. Enzyme-catalyzed processes in organic solvents. Biochemistry 82, 3192–3196.

Zaks, A., Klibanov, A.M., 1988. Enzymatic catalysis in nonaqueous solvents. J. Biol. Chem. 263, 3194–3201.

Zeng, W., Chen, H.Z., 2009. Air pressure pulsation solid state fermentation of feruloyl esterase by *Aspergillus niger*. Bioresour. Technol. 100, 1371–5.

Zhao, H., Zhang, X., Li, Z., 2001. Effects of periodic air pressure oscillation on growth of *Rhizobium leguminosarum* in a solid state fermenter. Process Biochem. 37, 165–167.